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In the normal breast, epithelial cells adhere to the connective tissue via junctions called hemidesmosomes. Using in vitro models, we have shown that hemidesmosomes play an important role in determining correct tissue architecture in the developing breast. They do so by the coordinated function of a matrix component (laminin-5) and its integrin receptors. Laminin-5 appears to direct hemidesmosome assembly as well as breast epithelial duct morphogenesis. To do so this matrix element requires precise proteolytic cleavage. We have also shown that hemidesmosomes are lost by breast epithelial cells which become invasive and this likely allows them to migrate through the breast connective tissue. The loss of hemidesmosomes in invasive cells is accompanied by down regulation of certain hemidesmosome components and modifications in others. For example, α6 integrin undergoes phosphorylation in invasive cells. In summary, during the course of our studies, we have provided novel insights into the role of hemidesmosomes in normal breast development as well as breast tumorigenesis.

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(5) Introduction

Modulation in adhesion of tumor cells compared with their normal counterparts plays an important role in development of invasive potential. Our application, as funded, concerned the fate of certain epithelial cell-matrix connectors called hemidesmosomes which are involved in anchoring breast epithelial cells to underlying matrix. During the course of this funded research, we have shown that a loss of hemidesmosomes appears characteristic of more invasive epithelial tumor cells. We have made use of our funding to analyze the expression and function of hemidesmosomes and hemidesmosomal components in breast epithelial cells (normal and tumor). In this progres report we detail the five major thrusts of our studies with each of these is being provided in a publication format.

(6) Body

The following were the set of tasks funded by the US Army in 1994. These are a revision of those tasks originally proposed in the Fall of 1993 and were approved by the US Army prior to funding.

Task 1. Immunochemical characterization of hemidesmosome components in normal and tumor breast tissue and their cultured cell counterparts
Task 2. Molecular analyses where message levels of HD proteins will be determined (complimentary to task 1).
Task 3. Electron microscopic evaluation of normal and tumor material
Task 4. Cell assays in matrigel

Tasks 1 and 3.

In the first year of the award we undertook studies revolving around tasks 1 and 3 i.e. expression of hemidesmosome components in normal and diseased breast tissue and cultured breast cells as well as electron microscopic evaluation of tissue and cell materials. These studies were published in Bergstraesser et al. (1995)(enclosed as part of the Appendix) and will be described first.

A. Hemidesmosomes in Normal and Tumor Breast Epithelial Cells

EXPERIMENTAL RATIONALE

A body of literature has been accumulating suggesting that adhesion molecules - those proteins and other substances that cells use to adhere to their substrate - may play a role in cancer cell invasion and metastasis (Schwartz, 1993; McIntyre et al., 1991). Data have suggested, on the one hand, that cancer cells may exhibit reduced adhesion molecule expression or function resulting in release of their substrate, and freeing cells to pile up or migrate. On the other hand, carcinoma cells may acquire expression of new adhesion

molecules to grip the basement membrane (BM) in order to traverse it, or to adhere to tissues at sites of metastasis (Brodt et al., 1990; Hand et al., 1985).

We and others have previously addressed this subject in the breast in studies of the integrin class of cell adhesion molecules, several of which are present in normal breast tissue, but reduced or absent in carcinoma (for example Bergstaesser and Weitzman, 1994; Pignatelli et al., 1992; D'Ardenne et al., 1991; Koukoulis et al., 1991; Zutter et al., 1990). Hemidesmosomes are another adhesion structure to study in this context. They are found only in epithelial cells which are usually stationary, and not in cells such as fibroblasts or macrophages which wander (for recent reviews see Owaribe et al., 1990; Jones et al., 1994; Legan et al., 1992; Schwarz et al., 1990; Green and Jones, 1996). Further, hemidesmosomes, more than other adhesion structures, may mediate firm, relatively immobile attachment to the BM, preventing the cell movement characteristic of invading malignant cells (Mutasim et al., 1985; Carter et al., 1990).

Hemidesmosome loss has been seen in cutaneous basal cell carcinoma (McNutt, 1976; Jones et al., 1989). In addition, the $\alpha 6\beta 4$ integrin is a component of the hemidesmosome (Jones et al., 1991; Stepp et al., 1990; Sonnenberg et al., 1991) and we and others found that the $\alpha 6$ and $\beta 4$ integrin subunits were not expressed in some malignant mammary epithelial cells (Bergstraesser and Weitzman, 1994; Pignatelli et al., 1992, D'Ardenne et al., 1991; Natali et al., 1992; Falcioni et al., 1988).

Although hemidesmosomes have been most commonly described in stratified epithelia, and the breast parenchyma is not normally stratified, ductal epithelia from various sources have been shown to contain hemidesmosomes (Schwarz et al., 1990). It is thought that epithelia which contain hemidesmosomes may be those under greater shear stress. The breast contains a ductal epithelium which undergoes great shear stress during lactation, and some electron microscope (EM) studies have suggested that at least breast myoepithelial cells, and perhaps luminal cells that contact the BM, may have hemidesmosomes (Tannenbaum et al., 1969; Ozello and Sanpitak, 1970; Stirling and Chandler, 1976; Franke et al., 1980; Williams and Daniel, 1983; Watson et al., 1988). It is not clear however whether these electron dense structures contain the same molecular components described in other well-studied hemidesmosomes.

In this paper we studied normal and malignant breast cells in tissue sections and in culture for the presence of hemidesmosomes and some of their constituent and associated proteins including the M_r 180 and 230 bullous pemphigoid antigens, a M_r 200 protein, and collagen VII. Normal human mammary epithelial cells (HMEC) had hemidesmosomes in vivo and in culture, and expressed the expected spectrum of hemidesmosome proteins, whereas invasive carcinoma cells lacked hemidesmosomes in vivo, and malignant cells in culture exhibited defects in hemidesmosome assembly. In addition we used normal HMEC in culture as a model to study hemidesmosome assembly and regulation.

MATERIALS AND METHODS

Tissues

Breast tissues were obtained from Northwestern Memorial Hospital, Evanston Hospital, or the Cooperative Human Tissue Network of the National Cancer Institute.

All malignant tissues used were infiltrating ductal carcinoma varying from grade I to III, and were from patients with from 0 positive lymph nodes to 10/10 positive level III nodes. Normal breast tissue was derived from such cancer patients, and from reduction mammoplasties. In addition, one sample of normal lactating tissue was obtained as the "normal" tissue from one of the mastectomies. Specimens were obtained fresh from surgery, and processed for EM, frozen sections, or cell culture.

Cell Culture

Mammary epithelial cells were derived from eleven infiltrating ductal carcinomas, seven reduction mammoplasties, and sites distant from two of the carcinomas. Epithelial or carcinoma cells were culled and grown on plastic by the method of Stampfer (1985) with revisions for tumor cell growth (Bergstraesser and Weitzman, 1993) in MCDB-170 medium (American Bioorganics Inc., Niagara Falls, NY) + serum-free supplements (29).

Mammary epithelial cell strains were determined to be epithelial by their expression of desmosome proteins by immunofluorescence (IF); and determined to be malignant by their ability to proliferate in the absence of certain growth factors, in the presence of transforming growth factor-β, and at high cell concentrations, and their inability to form three-dimensional structures on Matrigel BM-like substance (Bergstraesser and Weitzman, 1993).

804G cells (a rat urinary bladder carcinoma cell line) were maintained in culture as previously described (Riddelle et al., 1991; Langhofer et al., 1993). These cells were utilized for their capacity to produce laminin-rich extracellular matrix, as described below.

Immunofluorescence

Tissues fresh from surgery were snap frozen in liquid nitrogen and stored at -70^{0}C until use. Pieces of frozen tissue were embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN), sectioned on a Tissue-Tek cryostat (Miles Laboratories) to a depth of approximately 8 μm, and placed on poly-L-lysine (Sigma Chemical Company, St. Louis, MO)-coated glass microscope slides (VWR, Media, PA). Sections were fixed for five minutes in -20°C acetone (Mallinckrodt, Paris, KY) and air dried. Tissues from seven infiltrating ductal carcinomas, seven normal specimens, and one sample of normal lactating tissue from a cancerous breast were used.

Cultured cells were grown on glass coverslips (VWR) in six-well plates (Falcon, Lincoln Park, NJ) at 10^4 - 5 x 10^4 cells per well. Following three washes in phosphate buffered saline (PBS) pH 7.4 containing 0.2 g/liter KCl-0.2 g/liter KH_2PO_4-8.0 g/liter NaCl-1.15 g/liter Na_2HPO_4, cells on coverslips were fixed for three minutes in -20°C methanol (Fisher Scientific, Fair Lawn, NJ) and air dried. Cells from six carcinomas and four normal tissues (three reduction mammoplasties and one mastectomy) were used.

IF was performed as previously described (Bergstraesser and Weitzman, 1994). Slides were observed and photographed using a Leitz Laborlux D fluorescence microscope and TMAX 100 film (Eastman Kodak Co., Rochester, NY). All photographic exposures were for 1.5 min.

The following primary antibodies were used: serum from a bullous pemphigoid patient containing human autoantibodies reactive primarily with a M_r 230 plaque protein of the hemidesmosome (Klatte et al., 1989), 180 mouse monoclonal (Riddelle et al., 1993) and J17 rabbit polyclonal (Hopkinson et al., 1992) antibodies to a M_r 180 transmembrane hemidesmosome protein, 6A5 mouse monoclonal antibody to a M_r 200 hemidesmosome protein (Kurpakus and Jones, 1991), 9C3 mouse monoclonal antibody, and EBA human autoantibody (Kurpakus et al., 1990) to collagen VII anchoring fibril protein (which is also the epidermolysis bullosa aquisita antigen). Secondary antibodies included fluorescein-conjugated anti-mouse IgG + IgM, anti-human IgG + IgM and anti-human IgM; and rhodamine-conjugated anti-mouse IgG + IgM and anti-rabbit IgG (Kierkegaard and Perry, Gaithersburg, MD). Antibody concentrations were determined based on concentration curves.

Electron Microscopy

EM was performed using standard methodology (Hyat et al., 1986). Briefly, 1 mm^3 tissues fresh from surgery were fixed in 2.5% glutaraldehyde in 0.1 M Sorensens's phosphate buffer, postfixed in 1% OsO_4, stained in 2.5% uranyl acetate, dehydrated and infiltrated with propylene oxide and embedded in Spurr epoxy resin (all from Electron Microscopy Sciences, Fort Washington, PA). Thick sections were examined by a pathologist to confirm diagnoses and find regions of carcinoma. Twelve carcinomas and fourteen normal tissues (two from reduction mammoplasties and twelve from cancer patients) were used for EM.

Cultured cells were grown on glass coverslips or on Matrigel (Collaborative Research, Bedford, MA) -covered glass coverslips in six-well plates at 10^4 - 5 x 10^4 cells per well, processed similarly to tissue samples and embedded in Epon-araldite resin 812 (Tousimis, Rockville, MD; or Fisher Scientific). Samples from five malignant cell strains and five normal cell strains (four reduction mammoplasty patients' cells and one cancer patient's normal cells) were processed.

Thin sections were cut on a Reichert Ultracut E microtome (Reichert Instruments, Buffalo, NY), mounted on copper grids, stained with uranyl acetate and lead citrate (Electron Microscopy Sciences) and viewed at 80 kV in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA).

Growth of cells on Matrigel BM-like substance

35 mm^2 wells were coated with 500 µl undiluted cold Matrigel, then placed at 37· C for at least 30 minutes. Matrigel, a substance produced in vivo by the Engelbreth-Holm-Swarm murine tumor, resembles the lamina lucida portion of the BM, and contains (among many substances) mainly laminin, collagen IV, and proteoglycans.

Preparation of laminin-5-rich matrices from 804G cells

804G matrix was prepared as described previously (Langhofer et al., 1993). Briefly, 804G cells were grown to confluence on glass coverslips, the culture medium was aspirated, and the cells were washed in sterile PBS and removed from their matrix with 20 mM NH_4OH for five minutes followed by three PBS washes. HMEC were

plated on the matrix in their usual medium. After 24 hours, HMEC on 804G matrix were fixed in 2.5% glutaraldehyde and processed for EM as above.

RESULTS

In Vivo (Tissues)

Mammary epithelial cells in vivo contained electron dense hemidesmosomes at their basal plasma membranes where they were in contact with the BM (Fig. 1). These triangular-shaped plaques were associated with intermediate filaments intracellularly, and anchoring filaments and anchoring fibrils extracellularly. Hemidesmosomes were seen in all cells where contact with the BM could be shown. This included luminal cells that reached from the lumen to the BM (Fig. 1D,E) and myoepithelial cells that did not appear to reach the lumen (Fig. 1B).

In all intraductal regions of malignant tumors, cells in contact with the BM also exhibited apparently normal hemidesmosomes (Fig. 2). Some of these cells may have been normal myoepithelial cells, but many had morphological features of malignant cells and lacked the contractile filaments, large numbers of mitochondria and shape typical of myoepithelial cells. All cells in contact with the BM had hemidesmosomes, while cells not at the BM contained no hemidesmosomes, although numerous desmosomes and some adherens junctions were seen (Fig. 2).

In all invasive regions examined, no hemidesmosomes were seen in any cells (Fig. 3) whether single cells, in small groups, or invading en masse and attached to one another with desmosomes.

Because hemidesmosomes have not been well studied in the breast, we looked by IF microscopy at expression of several of the protein components of hemidesmosomes previously described in skin. The anchoring fibril protein collagen VII (Fig. 4C), M_r 180 (Fig. 5A) and M_r 230 bullous pemphigoid antigens, M_r 200 protein (Fig. 6C), and the $\alpha 6$ and $\beta 4$ integrin subunits (Bergstraesser and Weitzman, 1994) were all present in normal ducts at the basal aspects of cells ($\beta 4$ and the M_r 200 protein exhibited a basolateral distribution).

We next wished to determine whether abnormalities of hemidesmosome protein expression would be seen in breast carcinoma. Two patterns emerged. The first pattern, seen with collagen VII, the M_r 180 and 230 proteins and the $\alpha 6$ integrin subunit (Bergstraesser and Weitzman, 1994) is illustrated for collagen VII in figure 4. Staining for the hemidesmosome proteins was seen at the BM in normal ducts and in intraductal carcinoma. Cells piled up within cancerous ducts, and invasive cells exhibited no staining. A slight variation of this pattern was seen with the M_r 180 protein from which staining in all but one patient exhibited the aforementioned pattern, whereas the malignant cells from one patient exhibited no staining for the M_r 180 protein, even in regions of intraductal carcinoma (Fig. 5C,E).

The second pattern, seen with the M_r 200 protein and the $\beta 4$ integrin subunit (Bergstraesser and Weitzman, 1994) is illustrated in Figure 6. Staining was seen at the BM in normal ducts. In both carcinoma in situ and invasive carcinoma, regions of staining were seen and regions devoid of staining were also observed. For any one

patient there were regions of intraductal carcinoma outlined by staining, and intraductal carcinoma without staining, and invasive cells that stained or did not stain.

Cultured Cells

Because we saw abnormalities of hemidesmosomes and hemidesmosome protein expression in vivo, we decided to study cultured HMEC as a prelude to use of these cells for experimental manipulations.

Normal HMEC in culture exhibited electron dense, apparently normal, hemidesmosomes by two weeks in culture (Fig. 7). Malignant cell strains varied in their phenotype. For three patients, no cells could be found with hemidesmosomes; for one patient all cells had abundant, apparently normal hemidesmosomes; and the cells of one patient were a mix of cells with no hemidesmosomes, abundant hemidesmosomes, and a few hemidesmosomes.

We next stained cultured HMEC to see if the electron dense hemidesmosomes seen by EM might also contain the expected protein components. Normal HMEC expressed the anchoring fibril protein collagen VII in a basal secreted pattern (Fig. 8), and the M_r 200, 230 and 180 proteins in rows of basal tick mark-shaped plaques (Figs 9, 10). The $\alpha 6$ and $\beta 4$ integrins are also expressed in this manner and co-localize with one another (Bergstraesser and Weitzman, 1994).

Malignant HMEC also expressed all of the hemidesmosome-associated proteins tested for. However, several abnormalities of expression were consistently noted. When malignant cells were plated on coverslips, it took two to three weeks to see a basal secreted pattern of collagen VII staining, whereas normal cells showed this pattern in less than a week. In time course experiments (Fig. 8) we found that within one day normal HMEC produced antibody-detectable perinuclear intracellular collagen VII, whereas malignant cells had no immunostaining. At about seven days, malignant cells began to show perinuclear intracellular collagen VII staining while normal cells were already producing basal collagen VII in a secreted pattern.

The M_r 200 protein, on the other hand, continued to be expressed by malignant cells in only a dotted cytosolic pattern at incubations as long as 30 days (Fig. 9). The protein was never expressed basally by malignant cells. The M_r 230 and 180 proteins exhibited lines of basal tick-marks in malignant cells just as in normal cells and with the same time course (Fig. 10). As reported previously, the $\beta 4$ integrin subunit was also expressed identically in normal and malignant cells, whereas the $\alpha 6$ subunit was seen in only about 30% of malignant cells in culture (Bergstraesser and Weitzman, 1994).

Hemidesmosome assembly and regulation

Because normal HMEC formed hemidesmosomes in culture, we used them as a model to study hemidesmosome assembly. In time courses of hemidesmosome protein expression as determined by IF, all but one protein were seen intracellularly within 24 hours of plating, but a mature basal plaque-like distribution of proteins was seen with only $\alpha 6$ and the M_r 180 protein. Other proteins achieved their mature localization in a specific order. Hemidesmosomes were seen at the EM level for the first time at two weeks.

Because HMEC are highly mobile cells at low cell densities, and hemidesmosomes are structures involved in stable attachment; we wondered if assembly might be affected by cell density. In fact localization of the M_r 230, and 200 proteins, β4 and collagen VII were highly dependent on confluence. At 14 days in the majority of cells studied when they were at 25% confluence or less, the M_r 230 and 200 proteins and collagen VII were localized intracellularly, and β4 was not present, whereas at >40% confluence the four proteins were all present and basally located. On the other hand, α6 and M_r 180 protein localization were insensitive to cell density; these proteins were basally located in all cells at 14 days regardless of confluence.

Cell proliferation also influenced hemidesmosome assembly. We compared protein expression between three cell strains with rapid doubling rates and three cell strains with slow doubling rates by plating cell concentrations that would result in >50% confluence at two weeks. Rapidly proliferating cells did not localize the M_r 230 and 200 proteins, β4 or collagen VII basally after the usual two weeks, whereas slower growing cells did. α6 and M_r 180 protein localization were independent of doubling time as they were of cell density. Even rapidly proliferating cells promptly localized all expected proteins when they reached confluence.

Because motility and proliferation, which appeared to delay hemidesmosome protein expression, can reflect a de-differentiated phenotype, we wondered if differentiation might conversely stimulate hemidesmosome assembly. We plated normal HMEC on Matrigel, a BM-like substance that promotes HMEC morphogenesis and functional differentiation (Bergstraesser and Weitzman, 1993; Peterson et al., 1992). Electron dense hemidesmosomes were seen by EM at the bases of cells in fully formed three dimensional duct-like structures (Fig. 11), but not in cells on Matrigel that were still migrating to form these structures (not shown). The process of duct formation was complete at between 14 and 24 days. Likewise, hemidesmosomes were first seen between 14 and 24 days whenever duct formation was complete. Thus hemidesmosome formation on matrigel appeared to be differentiation-dependent rather than time-dependent.

Finally, because expression of hemidesmosomes in malignant cells in vivo appeared to correlate with the presence of BM proteins, we wondered whether ECM might actually stimulate hemidesmosome assembly. For this purpose we used the laminin-rich matrix produced by 804G cells and previously shown to induce rapid hemidesmosome formation in skin cells (Langhofer et al., 1993). Normal HMEC plated on this matrix formed electron dense hemidesmosomes within 24 hours instead of the usual two weeks (Fig. 12). This ECM does not promote HMEC morphogenesis.

SUMMARY AND DISCUSSION

In previous detailed EM studies of the breast, hemidesmosomes were noted at the BM in basal cells (Tannenbaum et al., 1969; Ozello and Sanpitak, 1979; Stirling and Chadler, 1976; Franke et al., 1980; Williams and Daniel, 1983; Watson et al., 1988). This information seems to have gone relatively unnoticed in the hemidesmosome literature, where hemidesmosomes continued largely to be discussed as characteristic of stratified epithelia. In addition, it was not clear from past studies whether

hemidesmosomes were unique to breast myoepithelial cells or used by all breast epithelial cells to attach to the BM.

In this study we clearly demonstrate that hemidesmosomes are found where all breast cells, whether myoepithelial or luminal, are in apposition to the BM. This strengthens the data adding breast epithelial cells to the expanding list of cell types that express hemidesmosomes, which now includes stratified epithelia such as skin, cornea, and esophagus (Owaribe et al., 1990; Owaribe et al., 1991); complex epithelia such as trachea, thymus, and transitional epithelia of the urinary bladder (Owaribe et al., 1990); glandular epithelia such as apocrine and salivary glands (Franke et al., 1980; Owaribe et al., 1990); and even the simple epithelium of the amnion (Robinson et al., 1984).

We would like to emphasize that we noted that luminal cells as well as myoepithelial cells contained hemidesmosomes. Since breast carcinoma cells tend to differentiate toward a luminal phenotype in, for example, expression of cytokeratins and actins, it is important to note that absence of hemidesmosomes from invading breast carcinoma cells does not merely represent loss of surrounding myoepithelial cells, but a clear downregulation of hemidesmosomes expression. Recently Clermont et al. (1993) studied collagen VII anchoring fibrils in the rat breast and also emphasized that both luminal and myoepithelial cells contain abundant hemidesmosomes in vivo.

We also determined that normal breast epithelium expressed at least six of the known hemidesmosome protein components, suggesting that hemidesmosomes have a similar structure and play a similar role in breast as they do in skin and other stratified tissues.

Hemidesmosomes are probably involved more in stable rather than motile adhesion. For example, $\alpha 6\beta 4$ was found only in nonmotile keratinocytes in culture (Carter et al., 1990), and hemidesmosomes are downregulated in epithelial cells that become motile to fill in a wound (for example Kurpakus et al., 1990; Kurpakus et al., 1991).

That hemidesmosomes are in fact adhesive structures used by normal epithelia to adhere to BM is suggested by wound healing studies in which re-epithelialized cornea can be easily lifted off until BM and hemidesmosomes have formed (Khodadoust et al., 1968). Further, the epithelium releases the stroma as a sheet in the genetic blistering diseases dystrophic epidermolysis bullosa, in which anchoring fibrils are congenitally absent (Briggaman and Wheeler, 1975; Epstein, 1992), and lethal junctional epidermolysis bullosa, in which hemidesmosomes are abnormal (Verrando et al., 1991); and in the acquired diseases epidermolysis bullosa aquisita and bullous pemphigoid, in which autoantibodies to collagen VII and to the M_r 230 and 180 hemidesmosome components, respectively, are found (Woodley et al., 1988; Stanley, 1992). Also, experimental addition of blocking antibodies to hemidesmosome proteins either in vivo or in culture causes loss of epithelial adhesion (Kurpakus et al., 1991).

The lack of hemidesmosomes in invasive breast carcinoma seen in this study may allow such cells to become less adherent to the BM. In fact during branching morphogenesis that occurs in the breast during embryogenesis, cells in the penetrating endbud lose expression of hemidesmosomes when they invade the stroma and branch into new ducts (Williams and Daniel, 1983; Clermont et al., 1993).

Interestingly, all tumors down-regulated the same hemidesmosome proteins at the same locations regardless of their stage or grade. There are two possibilities why this might be. 1) Certain steps in de-differentiation may typically occur in the same pattern. In fact, this apparent pattern relates to the normal pattern of assembly we saw in vitro. The proteins that assembled early (M_r 180, α6, collagen VII, and also M_r 230) appear to be co-regulated in carcinoma with BM, and may be the proteins that receive a signal from the BM to nucleate hemidesmosome formation in the normal breast. In fact α6 and M_r 180 are transmembrane proteins that probably play a role as extracellular matrix receptors. On the other hand, M_r 200 and β4 were lost in carcinoma independent of BM and were among the last to be expressed in normal hemidesmosome formation. Additionally, it was the late proteins that were apparently dependent on motility and proliferative rate for their assembly into hemidesmosomes.

2) Our sample size and exclusive use of ductal carcinoma may have biased our results; and it would be interesting in future studies to compare tumors of different types (eg. lobular, mucinous, ductal), stages, and grades in sample size with sufficient statistical power to detect differences between subgroups.

Another interesting observation in this study regards hemidesmosome expression in intraductal carcinoma in vivo. We noted hemidesmosomes in the basal cells by EM; however, by IF there were many regions in which the M_r 200 protein and β4 integrin were not expressed by basal cells. This suggests that even in intraductal carcinoma, the apparently ultrastructurally normal hemidesmosomes may actually be abnormal, and could be functionally impaired. Cells piled up within ducts and away from the BM in carcinoma in situ had no hemidesmosomes and lacked staining for component proteins. Abnormal downregulation of hemidesmosomes may allow these malignant cells to leave the BM. Alternately, if the BM regulates hemidesmosome expression, cells that have left the BM with hemidesmosomes intact may then downregulate hemidesmosome expression as the normal response to a loss of contact with the BM. This brings up the cause and effect question of hemidesmosome and BM expression: does the BM regulate hemidesmosome expression or vice versa?

Our in vitro results suggest that ECM stimulates hemidesmosome formation in HMEC. Hemidesmosome formation was accelerated from the 14 days seen on glass, to one day by culture on 804G cell matrix. A similar effect of matrix on hemidesmosome assembly has been previously noted when epithelial cell lines that did not form hemidesmosomes on plastic were able to make them on collagen I (Mann and Constable, 1977; Chapman and Eady, 1985; Eady, 1988). In addition, in embryogenesis, expression of BM clearly precedes hemidesmosome expression (for example, Fine et al., 1984; Gipson et al., 1988; Smith et al., 1988). However, most wound healing studies show that in epithelial cells that have migrated to fill a wound, hemidesmosomes reform before or simultaneously with BM (Kurpakus et al., 1990; Kurpakus et al., 1991; Croft and Tarin, 1970; Hintner et al., 1980; Stanley et al., 1981; Woodley et al., 1985; Sciubba, 1977; Gipson et al., 1989). In fact, BM initially reforms discontinuously beneath hemidesmosomes as if the hemidesmosomes nucleate BM formation (Briggaman and Wheeler, 1975; Briggaman et al., 1971; Akimoto et al., 1991).

It is not clear which of these models best reflects the situation in the normal breast or in breast cancer. In our study there appeared to be a correlation between localization of a cell at the BM and its expression of hemidesmosomes. Therefore the BM could be regulating hemidesmosome expression. However, some cells at the BM did not express every hemidesmosome protein, and some cells away from the BM did express some hemidesmosome proteins. We therefore feel that although lack of hemidesmosomes in invasive breast cancer could be due to a loss of BM, some hemidesmosome proteins are probably down-regulated independent of the BM. In addition, Wetzels, et al. (1991) found, as we did, that collagen VII was lost from almost all (94 of 97) invasive ductal carcinomas, but 13 of these retained staining for BM proteins, suggesting that loss of this hemidesmosome protein was not a result of loss of BM protein.

It is also possible that the invasive phenotype is an expression of de-differentiation in general such that both hemidesmosome and BM protein expression are downregulated by a preceding de-differentiation event. Such de-differentiation is seen in wound healing when epithelial cells migrate over stroma to re-epithelialize denuded regions, and hemidesmosomes and BM proteins are decreased in expression (Gipson et al., 1988; Hintner ert al., 1980) or at least relocated (Kurpakus et al., 1991). In addition, hemidesmosome formation appears to be linked to differentiation events in HMEC, as normal cells on Matrigel formed hemidesmosomes in a manner temporally linked with the differentiation that occurred on that matrix.

With respect to our observations on hemidesmosome formation in culture, most groups that have reported previously on cells cultured on plastic or glass have seen either no hemidesmosomes by EM, or "prehemidesmosomes" or "immature hemidesmosomes" (Thacher et al., 1991). Recently, however, a few rat and bovine cell lines able to make mature hemidesmosomes have been reported (for example Riddelle et al., 1991). In this work we report that our normal primary human cells form hemidesmosomes in culture containing the anticipated protein components. It should be noted that, subsequent to the work reporetd here we have also indentified a continuous human breast epithelial cell line which is also cpabale to assembling hemidesmosomes in vitro (Stahl et al., 1997; see below).

In time course studies of hemidesmosome protein expression, most hemidesmosome proteins were already being made as soon as cells were attached enough to be stained, but they were not all basally located into mature plaques, and EM-recognizable hemidesmosomes were not made until 2 weeks in culture. The order of assembly into mature plaques appears to be $\alpha 6$ and M_r 180 at day 1, then collagen VII, M_r 200 and 230, and finally $\beta 4$ at the time of final assembly. Kurpakus and her coworkers have outlined the order of hemidesmosome protein assembly in a wound healing tissue culture model, and they also see early appearance of $\alpha 6$, but accompanied by $\beta 4$ (Kurpakus and Jones, 1991). Other hemidesmosome proteins assemble in a somewhat variable order. It is somewhat surprising that $\alpha 6$ was expressed before $\beta 4$ in our model as these two integrin subunits pair to form one integrin molecule in hemidesmosomes. However, the $\alpha 6$ subunit has two possible partners, $\beta 1$ and $\beta 4$ to form two different integrins. We see expression of the $\beta 1$ subunit simultaneous to $\alpha 6$ on

day one but co-localization by IF of α6 and β4 by day 14 (Bergstraesser and Weitzman, 1994).

Except for the first two proteins to be localized basally, α6 and M_r 180, the hemidesmosome proteins studied remained intracellular while cells were mobile or highly proliferative. This suggests that these cells are able to couple hemidesmosome localization to machineries of cell cycle and motility. This is consistent with data in the skin in which epithelial cells proliferating and migrating to cover a wound contain hemidesmosome proteins intracellularly only, but assemble basal hemidesmosomes when migration and proliferation are complete.

Several of the parameters that appear to regulate hemidesmosome expression by normal HMEC are factors perturbed in carcinoma: motility, proliferative rate, BM synthesis. In fact, these interrelationships may play a role in the downregulation of hemidesmosomes we observed in malignancy. Several malignant breast cell strains in culture lacked hemidesmosomes by EM, however some malignant cells in culture did express hemidesmosomes. This heterogeneity probably reflects the heterogeneity of the tissues from which the cells were derived. For example, in cells grown from one tumor, some cells were seen with and others without hemidesmosomes by EM; these cells could represent intraductal and invasive cells respectively. In the one cell strain where we saw a normal complement of hemidesmosomes, only intraductal cells may have grown; and in the three cell strains with no hemidesmosomes, only invasive cells may be represented. It is also possible that cells derived from higher stage or grade tumors might exhibit the more abnormal phenotype. This could not be determined from the sample size in this study, but warrants further investigation.

Malignant cells in culture had a more normal array of hemidesmosome protein expression by IF than did malignant cells in vivo. As mentioned above, it is possible that the cells grown in culture were not fully representative of those found in vivo. For example, if a majority of the cells in culture were derived from the intraductal portion of carcinomas, a more normal phenotype would be observed.

A more interesting possibility is that hemidesmosome proteins not expressed in vivo can be reexpressed by tumor cells in a different milieu in culture. This suggests that the ability to express hemidesmosome proteins may not be lost by malignant cells; rather, hemidesmosome proteins may be downregulated in vivo by a program of altered differentiation which is partially reexpressed in culture. This was also suggested by the fact that malignant cells in vivo had such a complete change in hemidesmosome protein expression, when a loss of only one protein might have been expected if the loss were due to a mutation in that gene. It is further supported by the fact that collagen VII, which is eventually expressed basally in malignant cells in culture as in normal cells, exhibits a delayed conversion to the normal phenotype from that of intracellular expression. However, the fact that the M_r 200 protein remained abnormal in culture suggests the possibility of a permanent change in expression of this particular protein. Therefore, both the regulation of hemidesmosome proteins and some of the proteins themselves may be abnormal in breast cancer. This hypothesis will have to be tested further in subsequent studies.

As mentioned above, malignant breast cells in culture did exhibit some abnormalities of hemidesmosome protein expression by IF: the M_r 200 protein and collagen VII were seen intracellularly. This localization is reminiscent of wound healing, in which hemidesmosome proteins are also expressed intracellularly by epithelial cells migrating to close a wound (Riddelle et al., 1993). Some aspects of cancer invasion have been compared to wound healing, and internalization of hemidesmosome components could be a common mechanism by which epithelial cells become migratory.

In conclusion, in this paper we note the presence of hemidesmosomes in normal HMEC both in vivo and in culture containing the expected proteins, and a correlation of increasing downregulation of expression of hemidesmosomes with increasing aggressiveness of tumor cells. Such a correlation has been previously noted in other malignancies and with other adhesion molecules (reviewed in Albelda, 1993; Juliano and Varner, 1993; Fawcett and Harris, 1992; see also Berhens, 1994; Rucklidge et al., 1994). We expand their data to include hemidesmosomes and breast cancer, and suggest that normal HMEC may use hemidesmosomes to maintain their position in the mammary duct, and malignant cells may use downregulation of hemidesmosomes as a means of escape from usual tissue architectural restraints.

Further, our manipulations of cells in culture together with in vivo data suggest a model for hemidesmosome protein regulation. Early hemidesmosome protein localization may be signalled by the extracellular matrix and regulated by it, whereas localization of late hemidesmosome proteins (those usually localized to cell bases later) appear to be coupled to motility and proliferation. Both sets of proteins are affected in malignancy in which extracellular matrix, motility and proliferation are all abnormal. Since hemidesmosome expression appears to be linked to differentiation, this may reflect a program of de-differentiation by malignant cells in vivo which may be able to be partially reversed under certain circumstances (eg. in culture) suggesting that these malignant changes may be a combination of genetic and epigenetic events.

B. Identification of a Potential Novel Diagnostic for Invasive Breast Cancer Cells

EXPERIMENTAL RATIONALE

One of our motivations in carrying out the studies outlined under task 1 was to identify potential new antibody diagnostics for invasive breast cancer cells. In our search for such reagents, we have begun to extend the observations in Bergstraesser et al. (1995)(above) by investigating hemidesmosome integrin and hemidesmosome matrix components in normal and tumor breast tissue, with particular regard to laminin-5, a newly identified hemidesmosome component of basement membrane, and a receptor for laminin-5, namely the hemidesmosome integrin, $\alpha 6\beta 4$ (Jones et al., 1994). Some of our studies were premised on experiments in the laboratory which indicate that epithelial cell interaction with laminin-5 triggers a dephosphorylation of the $\alpha 6$ integrin subunit. These were published in Baker et al. (1997)(included in the Appendix).

MATERIALS AND METHODS

Cell Culture

804G cells were maintained in Dulbecco's Modified Eagles' Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum, penicillin (100 I.U./ml) and streptomycin (100 mg/ml). Conditioned medium was obtained from 804G cells one day post-confluency. FG cells were plated directly into 804G conditioned medium, or DMEM with 10% fetal calf serum, penicillin (100 I.U./ml) and streptomycin (100 mg/ml).

Antibodies

Monoclonal antibody GoH3 against the $\alpha 6$ integrin subunit was purchased from ImmunoTech (Westbrook, ME). The polyclonal antibody 6844, against the cytoplasmic domain of the integrin subunit $\alpha 6A$, were kind gifts from Dr. Vito Quaranta (Tamura et al., 1990). The mouse IgG1 monoclonal antibody 4E9G8 against $\alpha 6$ integrin subunit was purchased from ImmunoTech (Westbrook, ME). GB3, a mouse monoclonal antibody which recognizes the γ chain of human laminin-5, was obtained from Harlan Bioproducts for Science, Inc. (Indianapolis, IN) (Verrando et al., 1987, Matsui et al., 1995).

Tissue

Breast tissues were obtained from Northwestern Memorial Hospital, Evanston Hospital, or the Cooperative Human Tissue Network of the National Cancer Institute. All malignant tissues used were infiltrating ductal carcinoma varying from grade I to III, and were from patients with from 0 positive lymph nodes to 10/10 positive level III nodes. Normal breast tissue was derived from reduction mammoplasties.

Immunofluorescence

Sections of frozen breast tissue were prepared on a Tissue-Tek cryostat and placed on glass slides. Cells grown on coverslips and tissue sections on slides were processed for immunofluorescence as described in Klatte et al. (1989) and Riddelle et

al. (1992). Appropriate secondary antibodies were purchased from Southern Biotechnology (Birmingham, AL). For mouse/rat double labels, goat anti-mouse fluorescein antibodies pre-adsorbed against rat IgG and goat anti-rat rhodamine antibodies pre-adsorbed against mouse IgG were purchased from Southern Biotechnology. Coverslips or slides were viewed on an LSM10 confocal microscope or a Photomicroscope III (Carl Zeiss, Thornwood, NY). Unless stated, all images were taken within 0.5μm of the substrate attached surface of the cell. No adjustment of focus was made when changing lasers to analyze double labels. Images were stored on Sony magneto-optical disks (Inmac, Irving, TX) and printed on a Tek Phaser IISDX color printer (Tektronics, Beaverton, OR). For all immunofluorescence studies we use non-immune IgG as controls. This allows us to assess non-specific binding of fluorochrome conjugated secondary antibodies.

Immunoprecipitation

Cells were solubilized in Tris-buffered saline (TBS, pH 7.4) containing 0.5% NP-40, 2 mM CaCl, 1mM PMSF, 100 mM leupeptin, 1 mM pepstatin and 1 μg/ml aprotinin. After clarifying at 15,000 rpm for 15 mins at 4^0C, the supernatant was collected and precleared with protein-G agarose beads (GIBCO). The supernatant was then rotated at 4^0C with antibodies for 2 hr and then protein-G agarose beads were added for an additional 2 hr. The samples were centrifuged, the supernatant discarded, and the beads washed 3 times in a wash buffer (TBS containing 0.5% NP-40). After the final wash, the beads were boiled in Laemmli sample buffer (Laemmli, 1974) containing 10% β-mercaptoethanol (βME) for 5 mins.

For ^{32}P radiolabeling, subconfluent cultures of FG cells were washed and then incubated in phosphate free medium for 30 mins at 37^0C. Next, cells were passaged and replated into 60mm dishes in the presence or absence of laminin-5 in phosphate free medium. Immediately after plating, 0.1mCi/ml of [^{32}P] orthophosphate was added to each dish. After radiolabeling, cells were rinsed in TBS and subjected to immunoprecipitation as detailed above using GoH3 antibody. To inhibit phosphatase and kinase activity, 10mM sodium flouride, 4mM sodium orthovanadate, 10mM sodium pyrophosphate and 4mM EDTA were added to the immunoprecipitation lysis and wash buffers. Samples were analyzed by SDS-PAGE on 12% gels. Gels were either dried and exposed to film or separated proteins were transferred to nitrocellulose which was then processed for immunoblotting with 6844 α6A polyclonal antibodies. Autoradiographs and immunoblots were scanned and quantitated using Intellegent Quantifier (Bio Image, Ann Arbor, MI).

Peptide preparation

The peptide, NH_2-CIHAQPSDKERLTSDA-COOH, and phosphopeptide, NH_2-CIHAQP-pS-DKERLTSDA-COOH, were prepared commercially (Research Genetics, Huntsville, AL). These peptides match the amino acid sequence (residues 1036 to 1050) of a portion of the cytoplasmic domain of the α6A integrin subunit (Tamura et al., 1990). The amino terminal cysteine residue was included for future conjugation purposes. The phosphorylated serine in the second peptide corresponds to

serine residue 1041 which has been shown to be phosphorylated in vivo (Hogervorst et al., 1993). Peptides were spotted onto PVDF membrane (Bio-Rad, Richmond, CA) which was subsequently processed for immunoblotting as detailed below.

SDS-PAGE and immunoblotting

Whole cell extracts were made by solubilization of a confluent 100 mm dish of cells in 1 ml of Laemmli type sample buffer containing 10% βME (Laemmli, 1974). SDS-PAGE and immunoblotting were carried out as described previously (Zackroff et al., 1984; Klatte et al., 1989). Laminin-5 was purified from 804G conditioned medium as previously detailed (Baker et al., 1996). Approximately, 1.0 μg bovine serum albumin (BSA) was added to 5.0 μg of laminin-5 as a protein stabilizer.

RESULTS

Laminin-5 and the Phosphorylation State of α6 Integrin

Our interest in the use of antibodies specific for phosphoepitopes on α6 integrin was triggered by studies where we investigated the impact of culturing cells of the FG pancreatic carcinoma line in the presence of laminin-5 on the physiological state of α6 integrin (Baker et al., 1997). When FG cells are plated into laminin-5 rich medium, they "capture" laminin-5 and incorporate it into their matrix in an arc or circle type organization (Baker et al., 1996a).

For our studies we made use of monoclonal antibody 4E9G8 which has been reported to be specific for a phosphorylation sensitive epitope on α6A integrin subunit, the predominating splice variant expressed in FG cells, and rat monoclonal antibody GoH3 which recognizes the α6 integrin subunit irrespective of its phosphorylation state (Hogervorst et al., 1993). GoH3 antibodies stain FG cells, maintained under their normal culture conditions, in a streaky pattern towards their substratum attached surface (Fig. 13). In contrast, 4E9G8 antibodies fail to recognize comparable FG cells as determined both by immunoblotting and immunofluorescence (Figs. 14A,15A). Conversely, in FG cells incubated in laminin-5 containing medium, 4E9G8 antibody shows an arc or circle of staining towards the cell-substratum interface (Fig. 15C). This is the same pattern of organization seen using laminin-5 antibodies (Baker et al., 1996a). These arcs or circles co-localize with staining generated by GoH3 antibodies (Fig. 15E,F). In addition, 4E9G8 antibody reacts with the α6A "light" chain, migrating at 25kD, in extracts of FG co-incubated in laminin-5 containing medium (Fig. 14A).

To further define the epitope of 4E9G8 antibody, we generated peptides consisting of residues 1036-1050 of the α6A integrin subunit cytoplasmic domain. In one of our peptides, serine residue 1041 was phosphorylated. This peptide was not recognized by 4E9G8 antibodies unlike its non-phosphorylated counterpart (Fig. 14B). Both phosphorylated and non-phosphorylated peptides are recognized by a rabbit serum 6844 directed against the α6A integrin cytoplasmic domain (Fig. 14B).

To gain independent confirmation that the α6 integrin undergoes a dephosphorylation in FG cells maintained in the presence of laminin-5, we undertook an in vivo phosphorylation assay (Fig. 14C). The α6 integrin was immunoprecipitated using GoH3 antibodies from ^{32}P radiolabeled FG cells, maintained either in the absence or presence of laminin-5. Equal amounts of α6 integrin, precipitated from FG cells maintained under the two distinct conditions, were loaded onto gels as shown by the immunoblot using serum 6844. There is an apparent 37% decrease in the level of phosphorylation of the 25kD α6 integrin light chain in cells maintained in the presence of laminin-5 when compared with control cell immunoprecipitates (Fig. 14C).

Analyses of breast tissues for expression of laminin-5 and α6 integrin

The above studies led us to begin to study the phosphorylation state of α6 integrin in normal and tumor breast tissues. For our studies, normal breast tissues were obtained from reduction mammoplasties and invasive tumor breast tissue biopsies were surplus to pathological examination. Tissues were processed for double label immunofluorescence microscopy using a rat monoclonal antibody against a6 integrin (GoH3), which recognizes the α6 integrin subunit irrespective of its phosphorylation state, in combination with either laminin-5 antibodies (GB3) or antibody 4E9G8 against a phospho-epitope of α6 integrin. In normal breast tissue, 4E9G8 antibodies stain sites of epithelial cell/matrix interaction and co-localize with laminin-5 antibodies (Fig. 16). Similarly, GoH3 antibodies co-localize with GB3 antibodies (Fig. 16). In contrast, in invasive tumors, there is a loss of expression of laminin-5 at sites of tumor cell/matrix association and this correlates with a loss of staining by 4E9G8 antibodies but not by GoH3 antibodies (Fig. 16).

SUMMARY AND DISCUSSION

Our results indicate that there is a loss of laminin-5 in the matrix of invasive breast tumor cells. This is consistent with a loss of hemidesmosomes in such cells as determined by electron microscopy (Bergstraesser et al., 1995). In addition, loss of laminin-5 expression by tumor cells correlates with a dephosphorylation of the α6 integrin subunit.

We have only analyzed five invasive tumor specimens to date in the above manner. Nonetheless, our results suggest the exciting possibility that we can use a combination of the GoH3 and 4E9G8 antibodies to specifically mark invasive tumor cells i.e. cells which express phosphorylated α6 integrin may be more invasive. However, to validate this possibility, we now need to study non-invasive breast tumors as well as many more invasive tumor specimens. We also emphasize that we have not yet looked at the expression of laminin-5 at the leading edges of invasive breast tumors. Work on other tumor types suggests that laminin-5 may be expressed in the absence of formed hemidesmosomes in such tumor edges (Pyke et al., 1994; Pyke et al., 1995). The resolution of such issues are among our continuing goals.

Task 2

We have had some difficulty in obtaining appropriately preserved tissue to undertake the studies outlined under task 2 in the original proposal in which we had intended to analyze hemidesmosome message levels in normal and tumor breast tissues. In addition, we felt that the data that we would obtain would be descriptive and might provide only a modest incremental advance to our understanding of breast tumor progression. Thus we decided to modify our goals for this task and move in a new direction using the MCF-10A breast epithelial cell line in attempts to understand better both the function of the hemidesmosome matrix component laminin-5 as well as the relationship of laminin-5 structure to its variety of functions (see under task 4 where we have been able to show that the MCF-10A cell line provides an excellent model for our studies on hemidesmosomes and their components). We have made considerable progress in these new endeavors. In one direction, we have discovered a role for laminin-5 in the proliferation of MCF-10A cells, a continuous breast epithelial cell line as well as 804G cells. In a second new direction, we have shown that enzymatic processing of MCF-10A laminin-5 (in particular its α chain) has physiological consequences and determines whether laminin-5 can drive cell motility.

A. Laminin-5 and Proliferation

EXPERIMENTAL RATIONALE

Cell interaction with elements of the extracellular matrix impacts their adherence, motility as well as protein and gene expression (see for example, Adams and Watt, 1993; Roskelly et al, 1995). In intact, normal tissue, epithelial cells bind to extracellular matrix molecules which are organized into a complex multiprotein structure called the basement membrane. The major components of the latter include type IV collagen, proteoglycans and laminins. One laminin isoform, laminin-5, in particular plays an important role in establishing firm adherence of epithelial cells to the basement membrane since it is necessary for the assembly and maintenance of stable anchorage devices between epithelial cells and matrix called hemidesmosomes (Green and Jones, 1996; Baker et al., 1996b). However, recent reports also indicate that laminin-5 is expressed at the budding tips of certain invading tumor cells i.e. at sites where cancer cells are undergoing cell division but where there are most likely no hemidesmosomes (Pyke et al., 1994; Pyke et al., 1995). This provides an indication that laminin-5 has functions other than as an epithelial cell glue.

Two integrin receptors for laminin-5 have been identified. A variety of epithelial cells use the α3β1 integrin heterodimer to bind laminin-5 in vitro (Carter et al., 1991). However, this interaction appears to be transitory and, both in vivo and in vitro, cell interaction with laminin-5 at some point switches to the α6β4 integrin (Xia et al., 1996). Indeed, the latter association is apparently essential for both hemidesmosome assembly as well as the maintenance of the structural integrity of this cell-matrix adhesion device (Baker et al., 1996b; Jones et al., 1994; Borradori and Sonnenberg, 1996). Furthermore, laminin-5/α6β4 integrin complexes are believed to be conduits for signals from the external millieu of cells to the cytoplasm and potentially vice versa (Borradori and Sonnenberg, 1996; Mainiero et al., 1995; Giancotti et al., 1996).

A number of antibodies against laminin-5 have been shown to inhibit either cell adhesion and/or hemidesmosome assembly (Baker et al., 1996b; Carter et al., 1991; Rousselle et al., 1991). One such antibody CM6 recognizes the G domain of the α3 chain of rat laminin-5 (Baker et al., 1996b). Remarkably, we show here that the division of 804G rat epithelial cells treated with the antibody is compromised despite their attaching to and spreading on substrate. We have also investigated the impact of an antibody which inhibits human laminin-5 function on the breast epithelial cell line MCF-10A. MCF-10A cells, like 804G cells, secrete a laminin-5 rich matrix and assemble hemidesmosomes (Stahl et al., 1997). As is the case with 804G cells, laminin-5 antibodies inhibit proliferation of MCF-10A cells. Intriguingly, proliferation of antibody treated 804G and MCF-10A cells can be restored by providing them with exogenous laminin-5 but also, in the case of MCF-10A cells, by activation of β1 integrin.

MATERIALS AND METHODS

Cell Culture

804G cells were maintained as previsouly detailed (Riddelle et al., 1991). MCF-10A cells were obtained from American Tissue Culture Collection (Rockville, MD) and were maintained in a 1:1 mix of DME and Ham's F12 media supplemented with 5% equine serum, 0.01 mg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone.

Antibodies

Antibody CM6, which inhibits rat laminin-5 function, was described in Baker et al. (1996b). The human laminin-5 function blocking antibody P3H9-2 was purchased from Chemicon (Temecula, CA). The TS2/16.2.1 mouse hybridoma line was obtained from the American Tissue Culture Collection and hybridoma supernatant containing TS2/16.2.1 antibody was collected from subconfluent dishes of actively growing cells (van de Wiel-van Kemenade et al., 1992). The fluorescein conjugated anti-BrdU monoclonal antibody was purchased from Boehouringer Mannheim (Indianapolis, IN). The affinity purified rabbit anti-BM28 antibody preparation was described previously and was visualized in cells by indirect immunofluorescence using a fluorescein conjugated anti-rabbit immunoglobulin secondary antibody (Jackson Laboratories)(Todorov et al., 1994).

Matrix Molecules and Preparation of Rat and Human Laminin-5

Laminin-1, fibronectin and rat tail collagen were purchased from Collaborative Research (Bedford, MA). Laminin-1, fibronectin and rat tail collagen were coated onto cell supports according to the instructions of the supplier. Rat and human laminin-5 were prepared from 804G cell or MCF-10A cell conditioned medium respectively (Baker et al., 1996b; Stahl et al., 1997). In brief, cell medium was fractionated by cation exchange chouromatography. Fractions enriched in laminin-5 were further processed by anion exchange chouromatography and a final purification was achieved using hydroxyapatite chouromatography. This procedure will be detailed elsewhere (Fitchmun et al., manuscript in preparation).

Cell Proliferation and Cell Cycle Assays

Approximately $2X10^4$ 804G or MCF-10A cells were plated into wells of a 24 well plate in the presence of antibody or control IgG on various substrate supports. After 48 hours, the cells were trypsinized and then counted. For each experiment condition, thouree wells were assayed. For the bromodeoxyuridine (BrdU) assays, cells were plated onto glass chamber slides under various experimental conditions and 18 hours later, 10μm BrdU (Sigma Chemical Co., St. Louis, MO) was added directly to the cell culture medium. After 1 hour the cells were extracted in -20^0C methanol and allowed to air dry. DNA was subsequently denatured by incubating the extracted cells in 2M HCl at 37^0C for 1 hour. After washing in PBS, the cell preparations were

overlaid with a fluorescein conjugated monoclonal anti-BrdU antibody (Boehouringer-Mannheim, Indianapolis, IN) and incubated for 1 hour at 37^{0}C.

For visualization of BM28 antigen, cells were extracted as described in Todorov et al. (1995). In brief, cells grown on coverslips were washed in PBS containing 2 mM $MgCl_2$ and extracted in 0.5% Triton X-100, 20 mM Tris HCl pH 7.4, 100mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5 mM PMSF for 5 minutes at 20^{0}C. After washing in PBS the cells were fixed/extracted in methanol (-20^{0}C) followed by acetone (-20^{0}C) and processed for immunofluorescence using rabbit anti-BM28 antibodies. DNA was visualized by staining with 0.1 μg/ml 4,6-diamidino-2-phenylindole (DAPI). Fixed and stained cells were viewed using a Zeiss Photomicroscope III fitted with epifluorescence optics.

RESULTS

The Laminin-5 Antibody CM6 Inhibits Proliferation of 804G Cells

The mouse monoclonal antibody CM6 directed against the G domain of the α3 subunit of rat laminin-5 has been shown to destabilize certain cell-matrix connectors called hemidesmosomes as well as inhibit the ability of a laminin-5 rich to nucleate the assembly of hemidesmosomes (Baker et al., 1994). Here we have analyzed the proliferation of CM6 antibody treated 804G cells (Fig. 17). For these studies, a known number of cells was plated under various conditions and 48 hours later cells were collected and counted (Fig. 17). The index of division of CM6 treated cells is 38.6% of normal indicating that CM6 inhibits 804G cell growth (Fig. 17). To confirm the latter, we also assessed BrdU labeling of the antibody treated cells (Table 1). In this assay, only 12% of the CM6 treated cells stained compared with 44% of control IgG treated 804G cells (Table 1).

It should be noted that the CM6 antibodies do not prevent 804G cells attaching to or, in many instances, spreading onto their substrate (Fig. 18). In adddition, there is no significant detachment of cells during the course of the studies as determined by counting any floating cells in the medium from the antibody treated cell cultures each day. Furthermore, even though 804G cells fail to grow in CM6 containing medium, they are viable for up to ten days in the presence of antibody and could be induced to start to proliferate normally upon trypsinization and plating onto a fresh substrate in fresh medium (result not shown).

We next investigated whether CM6 antibody blocks the growth of 804G cells at a particular stage in the cell cycle. For these studies we made use of an antibody against BM28/hMCM2 which is a member of the recently defined family of MCM proteins thought to play an essential role in the regulation of DNA replication (Kearsey et al., 1996). The BM28 protein, as well as other members of the MCM family are found tightly bound to chouromatin during the G1 phase of the cell cycle and are gradually released during S phase (Todorov et al., 1995; Krude et al., 1996). BM28 is detected using BM28 antibody following mild detergent extraction prior to cell fixation. The BM28 antibodies generate distinctive nuclear stains which are dependent upon the phases of the cell cycle (Todorov et al., 1995). For example, cell nuclei are stained uniformly bright in G1 cells, show a spotty pattern in S phase cells, and are practically unstained in G2 and in mitotic cells. Thus BM28 staining, in combination with mild detergent extraction, provides a useful tool to visualize the cell cycle distribution of a given cell population (Todorov et al., 1995).

804G cells maintained in the presence of CM6 or control IgG antibody were extracted in mild detergent conditions and stained with BM28 antibody in combination with DAPI. The nuclei of both the CM6 and IgG treated cells show the full range of BM28 staining patterns (Fig. 19). Furthermore, the percentage of nuclei at different cell cycle stages as indicated by antibody staining was similar in 804G cell populations maintained in normal medium, in medium supplemented with CM6 antibody and in medium containing control IgG antibody (Table 2). This suggests that CM6 antibody treatment blocks cell growth randomly during the cell cycle.

Can growth of CM6 antibody treated 804G cells be restored by plating the cells on defined extracellular matrices including fibronectin (FN), laminin-1 (Ln 1), rat tail collagen (RTC) or human laminin-5 (hLn 5)(Figs. 17,20)? For these studies, we used hLn 5 which the rat specific CM6 antibodies fail to recognize (Baker et al., 1996b). Inhibition of division of 804G cells treated with CM6 antibody is not reversed by maintaining the cells on FN or Ln 1, the index of division being 24.7% and 38.2% of that observed in IgG treated control cell populations (Fig. 17). In contrast, the index of division of 804G cells treated with CM6 antibody is 84.7% and 72.5% of that of control IgG treated cells following plating onto RTC and hLn 5 coated substrates respectively (Fig. 17).

A Laminin-5 Antibody Inhibits Proliferation of MCF-10A Cells

To determine whether the above is phenomenon peculiar to 804G cells and /or the CM6 antibody we undertook comparable studies using cells from a different species (human) as well as a different function blocking laminin-5 antibody, namely the human specific antibody P3H9-2. Since CM6 antibody is rat specific, it could not be used in these particular studies (Baker et al., 1996b). The line we chose is MCF-10A which is derived from breast epithelium and which expresses laminin-5 in vitro (Stahl et al.,1997). Antibody P3H9-2 significantly reduces cell division of MCF-10A cells by 38.2% compared to IgG treated cells (Fig. 17). It does so with little, if any apparent, affect on the spreading of the cells onto their substrate after 24 hours (Fig. 18).

Only 24.4% of cells are stained by BrdU antibody in P3H9-2 antibody treated MCF-10A cultures compared with 57.9% cells stained in MCF-10A cells treated with IgG control antibody (Table 1). As is the case with CM6 treated 804G cells, the P3H9-2 antibody apparently blocks MCF-10A cells randomly in the cell cycle as determined using the BM28 antibody marker (Table 2). The effect of the P3H9-2 antibody is reversible and normal cell growth of antibody treated MCF-10A cells is restored by passaging the cells and replating them into fresh medium (result not shown).

The negative impact of antibody P3H9-2 on growth of MCF-10A cells is corrected by maintaining the cells on RTC and rat laminin-5 (rLn 5) but not by plating the cells LN-1 or FN (Figs. 1,4). MCF-10A cells maintained on RTC and rLn 5 in the presence of antibody P3H9-2 show growth of 86.5% and 107.4% respectively compared with IgG treated control cells (Fig. 17). In addition, P3H9-2 antibody inhibition of MCF-10A cell division is overcome by addition to the medium of the antibody TS2/16.2.1 which activates the β1 integrin subunit (Fig. 17)(van de Wiel-van Kemenade et al., 1992). In the latter instance, the index of growth is 105.8% that of control IgG treated cell cultures (Fig. 17).

SUMMARY AND DISCUSSION

Laminin-5 is now considered to play an important role in establishment of substrate attachment in a variety of epithelial cell types. One of the major pieces of evidence in support of this conclusion comes from recent analyses of the human inherited blistering skin disease, junctional epidermolysis bullosa (JEB), and the autoimmune disease cictricial pemphigoid (CP). In certain families afflicted by JEB there are defects in or a loss of laminin-5 (reviewed in Borradori and Sonnenberg, 1996). The latter result in the dysadhesion of keratinocytes thereby bringing about blistering in the epidermis in the region of keratinocyte-extracellular matrix interaction. In CP, autoantibodies against laminin-5 appear to be pathogenic and antibody binding to the basement membrane zone induces epithelial blistering (Domloge-Hultsch et al., 1992).

Yet even though laminin-5 is clearly involved in adhesion, its expression at the leading edges of migrating tumor populations suggests that this heterotrimer may play roles in cell motility and cell proliferation (Pyke et al., 1994; Pyke et al., 1995). There is already published evidence that laminin-5 can enhance epithelial cell motility (Zhang and Kramer, 1996). Here we show that laminin-5 impacts cell proliferation. Using an immunological approach we have inhibited the function of laminin-5 produced by two distinct cell lines generated from two different organs of two different species. In both instances, the division of cells in which we have "blocked" laminin-5 function is significantly inhibited. In addition, the cells are blocked apparently randomly in the cell cycle.

CM6 antibody treated 804G cells remain somewhat rounded compared with their control counterparts although they are able to attach and spread onto their substrate. P3H9-2 antibody treatment of MCF-10A cells has no obvious effects on MCF-10A cellular morphology. The cells appear to spread fully onto their substrate in the presence of the blocking antibody. This indicates that the inhibition of cell division induced by blocking laminin-5 in both 804G and MCF-10A cells is not a secondary consequence of the loss of cell-substrate contact which is known to trigger the differentiation of many epithelial cell types (see, for example, Adams and Watt, 1989). Rather it is the result of a block in some type of signal "encoded" by laminin-5 which is transduced via cell surface receptors to the overlying cells and which can directly modulate the progress of the cell cycle.

Proliferation of both CM6 antibody treated 804G and P3H9-2 antibody treated MCF-10A cells can be restored to normal levels not only if they are provided with exogenous laminin-5 but when they are plated onto rat tail collagen suggesting that the later matrix molecule can substitute for laminin-5. This result gave us a clue to the nature of the receptor involved in laminin-5 regulation of cell growth since cell interaction with both these particular matrix molecules can be mediated by the $\alpha 3\beta 1$ integrin heterodimer (Carter et al., 1990; Hynes, 1992). The use of human cells enabled us to further this aspect of our study since we could assess whether a well characterized $\beta 1$ integrin antibody which activates its human antigen in the absence of ligand could rescue the proliferation of MCF-10A treated with a blocking laminin-5 antibody (van de Wiel-van Kemenade et al., 1992). The integrin activating antibody

TS2/16.2.1 does so, supporting the idea that the mitogenic effects of laminin-5 are transduced to the cell cycle machinery of cells via a β1 containing integrin receptor, most likely the α3β1 heterodimer. Whether the latter integrin "modulates" cell growth by way of tyrosine kinases, a MAP kinase, a G protein and/or the Ras pathway will be the object of our future studies (Rosales et al., 1995).

The idea that laminin-5 and its cell surface receptors, has an impact on cell proliferation is consistent with studies on laminin-1 which indicate that it also may impact cell growth (Panayotou et al., 1989; Mortarini et al., 1995). For example, laminin and β1 integrins exert a mitogenic effect on human melanoma cells (Ocalan et al., 1988). Laminin-1 has also been shown to stimulate the proliferation of myoblasts and bone marrow derived macrophages in a dose dependent manner while human thymocytes proliferation is apparently influenced by laminin/merosin and their receptors, α3β1 and α6β1 (Ocalan et al., 1988; Ohki and Kohashi, 1994; Chang et al., 1995). In addition, the β1 cytoplasmic domain has been reported to modulate cell proliferation thourough an integrin signaling pathway (Pasqualini and Helmler, 1994).

Both laminin-5 antibodies used in this study recognize the α3 subunit of the heterotrimer and one of these antibodies has been localized to the G-domain of the intact laminin-5 molecule (Baker et al., 1996b). Thus we speculate that the proliferative impact of laminin-5 is encoded by a sequence of amino acids in or close to the G domain of the molecule. Furthermore, based on our observations, we propose that expression of laminin-5 at the leading tip of epithelial tumor populations in vivo enhances cell proliferation and thereby can drive tumor growth. We are currently evaluating such a possibility in breast tumor tissue specimes. In normal wound healing a similar phenomenon may also occur in which the deposition of laminin-5 at the edge of migrating epithelial cells triggers cell division.

B. Laminin-5 Processing and Its Physiological Consequences

In this second part of the substituted task 3, we posed the following question. Does enzymatic processing of the subunits of laminin-5 determine laminin-5 functions? The answer to this question could be crucial to our understanding of the development of invasive phenotype.

EXPERIMENTAL RATIONALE

Epithelial cells are separated from connective tissue by a basement membrane, which is composed of a variety of extracellular matrix molecules including proteoglycans, collagen and laminin isoforms. Together these proteins create a framework that is essential for maintaining tissue integrity. However, extracellular matrix proteins play more than just a structural role. They also display a diverse set of biological functions that regulate adhesion, migration, proliferation, differentiation and gene expression of adjacent cells (Roskelly et al., 1995).

Laminin, of which there are at least ten isoforms, is a major component of basement membranes and has been shown to mediate cell-matrix attachment, gene

expression, tyrosine phosphorylation of cellular proteins, and branching morphogenesis (see, for example, Streuli et al., 1995; Malinda and Kleinman, 1996; Timpl and Brown, 1994; Tryggvason, 1993; Stahl et al., 1997). The expression patterns of the laminin isoforms are tissue specific. The laminin-5 isoform (nicein, epiligrin, kalinin) is abundant in transitional epithelium, stratified squamous epithelia, lung mucosa and other epithelial glands (Kallunki et al., 1992; J.C.R. Jones, unpublished observations). Laminin-5 is a heterotrimer consisting of α3, β3 and γ2 subunits that associate via large α-helical regions to produce a cruciform-shaped molecule (Rousselle et al., 1991; Baker et al., 1996b). Laminin-5 is synthesized initially as a 460 kD molecule, which undergoes specific processing to a smaller form after being secreted into the extracellular matrix (Marinkovich et al., 1992; Matsui et al., 1995a; Vailly et al., 1994). The size reduction is a result of processing of the α3 and γ2 subunits from 200-190 to 160 kD and from 155 to 105 kD respectively (Marinkovich et al., 1992; Matsui et al., 1995a; Vailly et al., 1994). The identity of the proteases involved in such proteolytic events has not been described previously.

In a number of studies, it has been demonstrated that laminin-5 produced by 804G cells can nucleate the assembly of hemidesmosomes by SCC12, HaCaT and pp126 cells as well as corneal cells maintained in vitro (Langhofer et al.,1993; Hormia et al., 1995; Baker et al., 1996a; Baker et al., 1996b; Tamura et al., 1997). However, SCC12, HaCaT and pp126 cells themselves secrete laminin-5 which is incapable of supporting the assembly of hemidesmosomes, suggesting that structural differences between laminin-5 molecules may regulate their functions. The current data demonstrate that the electrophoretic mobility of the α3 chain of laminin-5 in the matrix of these cell types is distinct. Furthermore, proteolytic processing of the α3 subunit of laminin-5 in the extracellular matrix is crucial for hemidesmosome formation. We also present evidence that processing of the α3 subunit of laminin-5 is likely mediated in vivo by a plasmin-dependent mechanism involving tissue-type plasminogen activator (tPA)-catalyzed plasminogen activation. A model is proposed that could explain the role of laminin-5 processing in hemidesmosome assembly.

MATERIALS AND METHODS

Protein Preparations and Other Chemicals

Plasminogen and plasmin were purified as previously described (Stack et al., 1993, 1994a, 1994b). Purified two-chain tissue-type plasminogen activator (tPA) was the generous gift of Dr. Henry Berger, Wellcome Research Laboratories, Research Triangle Park, NC. Urinary-type plasminogen activator (uPA) was purchased from Calbiochem (La Jolla, CA). The serine proteinase inhibitor, dichloroisocoumarin (DCI), was purchased from Sigma Chemical Company (St. Louis, MO). Matrix metalloproteinase-2 (MMP-2, gelatinase A) and MMP-9 (gelatinase B) were obtained from the serum-free conditioned medium of epithelial ovarian carcinoma cells as previously described or were the generous gift of Dr. Hideaki Nagase of the University of Kansas (Young et al., 1996).

For affinity purification of pp126 laminin-5, tissue culture plastic was coated with 50 μg/ml GB3 antibody in 10 mM Tris, pH 7.4, overnight at 4°C. Dishes were rinsed briefly, then incubated with conditioned medium from pp126 cells for one hour at 37°C. The dishes were then washed in 20 mM Tris pH 7.4 containing 250 mM NaCl, followed by three washes in 10 mM Tris pH 7.4. For overlay studies, laminin-5 purified from MCF-10A cells was provided by Desmos Inc. (San Diego, CA).

Cell culture and preparation of laminin-5 matrices

MCF-10A cells and 804G cells were maintained as described previously (Stahl et al., 1997; Riddelle et al., 1991). Normal human epidermal keratinocytes (NHEK, purchased from Clonetics Corp., San Diego, CA), SCC12 and pp126 cells were maintained in the serum-free growth medium Keratinocyte-SFM (Gibco BRL, Gaithersburg, MD) supplemented with 20 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 ng/ml EGF, and 50 μg/ml bovine pituitary extract. MCF-10A, pp126, SCC12, NHEK and 804G cell matrix were prepared as described previously (Gospodarowicz, 1984; Langhofer et al., 1993). Laminin-5 comprises at least 80% of the protein in such matrix preparations. In experiments where various proteases were used, pp126 cells were removed from their matrix with ammonium hydroxide as previously described. Then the matrix was extensively washed in PBS and approximately 50 μg of matrix was incubated with 1 ml of PBS containing plasmin at concentrations of 0.01, 0.1 and 1 μg/ml for 90 min at 37°C or 50 μg of matrix was treated with 1 ml of PBS containing 5 μg/ml of either MMP-2 or MMP-9 overnight at 37°C. In some studies, 50 μg of pp126 cell matrix was treated overnight at 37°C with 1 ml of PBS containing either 5 or 10 μg/ml tPA. In all cases, DCI was then added to the treated matrix at 10 μg/ml, for 15 minutes at room temperature. After washing with PBS, the treated matrices were solubilized in sample buffer consisting of 8 M urea, 1% sodium dodecyl sulfate in 10 mM Tris-HCl, pH 6.8, and 15% β-mercaptoethanol.

SDS-PAGE, Western Immunoblotting and Overlay Assays

Protein preparations were separated by the method of Laemmli (1970) in 6% acrylamide gels, then transferred to nitrocellulose (Harlow and Lane, 1988). The nitrocellulose membranes were processed for blotting as described in Zackroff et al. (1984). Blots were developed either using chloronaphthol as a colorimetric reagent, or using the LumiGlo chemiluminescence kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD). For overlay assays, 1 ng of protein was dotted onto nitrocellulose, which was then blocked in PBS containing 0.2% fish gelatin. The membrane was subsequently incubated overnight with 20 μg/ml of either tPA, uPA or plasminogen, at 4°C with vigorous shaking. The nitrocellulose was then blocked in 5% milk in PBS and processed for immunoblotting.

Antibodies

The mouse monoclonal antibody against the γ2 chain of human laminin-5, GB3, was purchased from Harlan Bioproducts for Science, Inc., Indianapolis, IN (Verrando et al., 1987; Matsui et al., 1995b). For production of a rabbit serum against the human γ2 chain, a clone encoding amino acids 522 through 722 of human laminin-5 γ2 chain was identified in a λgt11 keratinocyte expression library (Clontech Labs, Inc., Palo Alto, CA) as detailed in Langhofer et al. (1993). A 25ml culture of Y1090 was grown at 37°C in shaking suspension to an OD of 0.7 and was subsequently inoculated with 10^8 phage containing the laminin-5 γ2 insert. After one hour, IPTG was added to a concentration of 1 mM and incubated for an additional three hours. Cells were pelleted and resuspended in gel sample buffer (8 M urea, 1% SDS in 10 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol). The resulting protein sample was then processed for SDS-PAGE, simultaneously with a protein sample derived from non-IPTG-induced cells. Following staining of the gel, a prominent protein migrating at about 140 kD (i.e. a portion of laminin-5 γ2 chain fused with β-galactosidase) was observed exclusively in the induced cells. This polypeptide was excised, rinsed in PBS and then used to immunize a rabbit for polyclonal antibody production (Harlow and Lane, 1988). Blood was collected from the rabbit at three week intervals.

Clone 17, a mouse monoclonal antibody specific for the β3 chain of human laminin-5, was purchased from Transduction Laboratories (Lexington, KY). The mouse monoclonal antibody 10B5, which recognizes the α3 subunit of rat and human laminin-5 was generated using 804G laminin-5 as immunogen as described in Langhofer et al. (1993). J21 antiserum was prepared against MCF-10A matrix and shows reactivity with all three laminin-5 subunits but does not recognize other laminin isoforms or fibronectin (Jones, unpublished observation). PAM-3, a mouse monoclonal antibody against human tPA, and monoclonal antibody #394 against human uPA, were purchased from American Diagnostica Inc. (Greenwich, CT). Anti-human plasminogen rabbit antiserum was described in Stack et al. (1993, 1994a and 1994b).

Immunofluorescence

MCF-10A and pp126 cells were maintained on glass coverslips for 24-48 hours, then permeabilized in acetone at -20°C for two minutes and air dried. Coverslips were incubated with a mix of primary antibodies diluted in PBS at 37°C in a humid chamber for one hour, washed three times in PBS, and incubated for an additional hour at 37 °C with the appropriate mix of secondary antibodies conjugated to rhodamine and FITC. Mounted glass coverslips were viewed using a Zeiss LSM10 laser scanning confocal microscope (Zeiss Inc., Thornwood, NY). Images were stored on Sony optical discs and printed on a Tektronix printer (Tektronix, Wilsonville, OR).

Electron microscopy

Cells were maintained on tissue culture plastic or on matrix derived from pp126 or MCF-10A cells prepared as described above. In some cases, the matrix was treated with purified plasmin or tPA, as described above, before addition of cells. After 36 hours, the cells were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for a minimum of 30 minutes. Fixed preparations were washed three times in 0.1 M sodium cacodylate buffer, pH 7.2, and post-fixed in 1% osmium tetroxide containing 0.8% potassium ferricyanide. Samples were then stained with uranyl acetate, dehydrated six times in ethanol, and embedded in Epon-Araldite resin (Tousimis Corp., Rockville, MD). Sections were cut perpendicular to the substratum and viewed on a JEOL 100CX electron microscope at 60 kV (Riddelle et al.,1991).

Motility Assay

Cells were plated in media containing 20 mM HEPES onto affinity-captured laminin-5 or laminin-5-rich matrix (see above) for 1 hour. They were subsequently maintained at 37°C and viewed by phase contrast microscopy using a Nikon Diaphot system. The field was photographed every five minutes over a two hour period with a chilled CCD camera (Hamamatsu Photonic Systems Corp., Park Ridge, IL). The location of each cell was translated to numerical coordinates using the public domain NIH Image Program, and motility for each cell was calculated as displacement in micrometers from the starting point to the ending point. An average of thirty cells was monitored for each trial.

RESULTS

The α3 subunit of laminin-5 is processed differently in diverse cell types

The rat epithelial cell line 804G and the human breast epithelial cell line MCF-10A are unusual in that both assemble cell-matrix attachment devices called hemidesmosomes, when maintained in tissue culture (Riddelle et al., 1991; Stahl et al., 1997). Recent data indicates that this property is dependent upon the laminin-5 secreted by these cells (Baker et al., 1996b; Stahl et al., 1997; Tamura et al., 1997). However, laminin-5 is expressed by many other cell types, such as the squamous cell carcinoma line SCC12 and the transformed oral epithelial cell line called pp126, which do not assemble hemidesmosomes under normal conditions (Langhofer et al.,

1993; Baker et al., 1996b; Tamura et al., 1997). In addition to expressing laminin-5, both SCC12 and pp126 cells express all the major known protein components of hemidesmosomes, yet only assemble hemidesmosomes when plated onto laminin-5 secreted by 804G cells or MCF-10A cells (Langhofer et al., 1993; Baker et al., 1996b; Tamura et al., 1997; Jones, unpublished observations). The latter finding suggests the possibility that distinct structural and functional forms of laminin-5 may be expressed by different cell types. Thus we have analyzed the subunit composition of laminin-5 secreted by 804G, MCF-10A, pp126, SCC12 cells, as well as a "normal" cell population (NHEK), by Western immunoblotting using a panel of antibodies against specific laminin-5 subunits.

An antibody against the β3 subunit of laminin-5 recognizes a band migrating at 145 kD present in MCF-10A as well as SCC12, NHEK and pp126 cell matrices (Fig. 21a). This particular monoclonal antibody does not recognize rat material, hence the lack of reactivity with the 804G cell matrix preparation. A polyclonal antibody recognizing the γ2 subunit of human laminin-5, which displays cross-reactivity with the rat homologue, detects 155 and 105 kD polypeptides in the matrices of MCF-10A, pp126 and NHEK cells but only a 105 kD species in the matrices of 804G and SCC12 cells (Fig. 21b). For the analysis of the α chain of laminin-5, we made use of a monoclonal antibody, 10B5, which was prepared against laminin-5 in the extracellular matrix of 804G cells (Langhofer et al., 1993; Baker et al., 1996b). 10B5 specifically recognizes the α3 chain of rat laminin-5, and displays cross-reactivity with the α3 subunit of the human homologue (Fig. 21c). The α3 subunits of pp126, SCC12 and NHEK cell laminin-5 migrate at 190 kD, identical to the reported size of the unprocessed α3 chain of laminin-5 (Fig. 21c)(Marinkovich et al., 1992; Matsui et al., 1995a). The appearance of the unprocessed α3 chain of laminin-5 in NHEK matrix has not previously been noted (Marinkovich et al., 1992; Matsui et al., 1995a). In contrast, in the matrices of MCF-10A and 804G cells the α3 chain migrates at 160 kD, similar to the molecular weight of the processed α3 chain subunit (Fig. 21c)(Marinkovich et al., 1992; Matsui et al., 1995a).

These results indicate that there is no obvious correlation between the sizes of the γ2 or β3 subunits of laminin-5 and the ability of a cell to assemble a hemidesmosome. In contrast, the matrix of those cells (MCF-10A and 804G) that assemble hemidesmosomes contains processed α3 subunits while matrix of those cells (pp126, SCC12 and NHEK) that do not assemble hemidesmosomes contains unprocessed α3 subunits. Thus we next tested several proteinases that are known to cleave extracellular matrix proteins for their ability to alter the mobility of the laminin-5 α3 subunit of pp126, SCC12 and NHEK cells. For these studies, we chose proteinases that are expressed by these cells (i.e. plasmin, MMP2 and MMP9)(Stack, unpublished observations). We describe only those results using pp126 cells since they are identical to those using the other cell lines. MMP-2 and MMP-9 at enzyme-substrate ratios of about 1:10 exhibit no obvious effect on the α3 subunit of pp126 matrix (not shown). We also treated approximately 50 μg of pp126 matrix with 1 ml of PBS containing the proteinase plasmin at concentrations of 0.01, 0.1 and 1 μg/ml for 90min. Plasmin at concentrations of 0.01 and 0.1 μg/ml has no obvious effect on

the subunits of laminin-5 (result not shown). However, following treatment of pp126 matrix with plasmin at 1μg/ml, the α3 subunit is converted to a 160 kD species, as shown by Western immunoblotting with 10B5 antibody (Fig. 21c). This treatment does not induce proteolysis of the β3 and γ2 subunits (Fig. 21a,b).

Functional consequences of processing of the α3 subunit of laminin-5
a) Cell motility

The specific limited-proteolytic cleavage of the α3 chain of pp126 cell laminin-5 from 190 to 160 kD suggests that plasmin-mediated proteolysis may have functional consequences for this matrix ligand. Thus we assessed the motility of SCC12 cells plated on the laminin-5-rich matrix of pp126 cells relative to MCF-10A matrix. SCC12 cells plated onto MCF-10A matrix exhibit significantly lower motility after two hours compared with SCC12 cells plated onto pp126 matrix (Fig. 22). However, SCC12 cells plated onto plasmin-modified pp126 matrix display a 2.5 to 3 fold decrease in motility, similar to that observed on MCF-10A matrix (Fig. 22). SCC12 cells plated onto plasmin-treated laminin-5, affinity purified from pp126 cell conditioned medium, also showed a decrease in motility compared with SCC12 cells plated onto affinity purified, untreated pp126 cell laminin-5 (Fig. 22). It should be noted that the differences in motility of SCC12 cells on these distinct matrices are statistically significant as determined using the non-parametric ANOVA Mann-Whitney U test. For example, the difference in motility of SCC12 cells on affinity purified laminin-5 compared with that on plasmin-treated affinity purified laminin-5 shows significance at $p < 0.008$.

b) Hemidesmosome Assembly

Laminin-5 is the extracellular ligand of the integrin pairs α6β4 and α3β1 (Carter et al., 1991; Niessen et al., 1994). The precise physiological role of α3β1 integrin-laminin-5 ligation is unknown, whereas the α6β4 integrin-laminin-5 complex forms the core of hemidesmosomes (Stepp et al., 1990; Jones et al., 1994; Green and Jones, 1996; Borradori and Sonnenberg, 1996). We therefore investigated whether SCC12 cells are induced to assemble hemidesmosomes on either untreated pp126 cell laminin-5-rich matrix or plasmin-modified pp126 cell matrix. SCC12 cells were plated onto these matrices and after 36 hours the samples were processed for electron microscopy. SCC12 cells maintained on untreated pp126 matrix assemble few hemidesmosomes at their basal surface (average = 0.2 hemidesmosomes/cell; Fig. 23a). In contrast, SCC12 cells plated onto plasmin-modified pp126 matrix readily assemble "mature" hemidesmosomes (average = 2.3 hemidesmosomes/cell). These hemidesmosomes possess triangular-shaped electron-dense cytoplasmic plaques, fine anchoring filaments, sub-basal dense plates and are associated with the keratin intermediate filament cytoskeleton (Fig. 23b).

Plasminogen and tPA interaction with laminin-5
a) In vivo association

The apparent ability of plasmin to modify the structure of laminin-5 and the resulting physiological consequences on cell behavior raise the question of how those events are regulated in vivo. Plasmin is generated by cleavage of the proenzyme plasminogen, which is present in serum and found in association with extracellular matrices. The cleavage of plasminogen to produce the functional enzyme plasmin is mediated by either of two so-called plasminogen activators designated tissue-type (tPA) and urinary-type plasminogen activator (uPA)(Wun, 1988). Since colocalization of enzyme and substrate is an important regulatory property governing matrix remodeling, we determined whether plasminogen and either tPA or uPA are associated in vivo with laminin-5 (Ranby, 1982; Stack et al, 1995).

MCF-10A and pp126 cells were processed for double-label immunofluorescence microscopy using an antiserum raised against purified plasminogen or tPA in combination with antibodies against human laminin-5 (Fig. 24). Laminin-5 antibodies generate staining patterns in 'circles and arcs' at the basal aspect of the cells (Fig. 24a,d,g,j). In addition, some staining is also seen in areas of the substrate not covered by cells, as reported previously (Rousselle et al.,1991; Baker et al., 1996a; Stahl et al., 1997). Interestingly, plasminogen staining in both MCF-10A and pp126 cell cultures colocalizes almost exactly with laminin-5 (Fig. 24a,b and d,e). In MCF-10A cells, a monoclonal antibody against tPA shows an almost identical staining pattern to that generated by an antiserum against laminin-5 (Fig. 24g,h). In contrast, tPA does not localize to the matrix of pp126 cells although it shows weak staining of cell bodies (Fig. 24j,k). The cell bodies of pp126 cells but not matrix are also stained by a uPA antibody while the cells show no staining with an antibody against uPAR (result not shown). MCF-10A cells and underlying matrix do not stain positively for uPA or uPAR, suggesting that uPA is not involved in plasmin-mediated laminin-5 processing in vivo, at least in this cell type (result not shown).

The above results suggest the possibility that lack of tPA in the matrix of pp126 cells may preclude conversion of plasminogen to plasmin and the subsequent plasmin-mediated α3 chain processing in pp126 cell matrix. We therefore determined whether addition of purified tPA to pp126 matrix could induce cleavage of the pp126 cell α3 subunit. Western immunoblotting of the tPA-treated pp126 matrix reveals that the laminin-5 α3 subunit is processed to a 160 kD species which comigrates with the laminin-5 α3 subunit in MCF-10A matrix (Fig. 25).

b) In vitro interaction

The observed colocalization of tPA and plasminogen with laminin-5 in fixed MCF-10A cell matrix suggests that both might bind directly to laminin-5. This was confirmed using a dot blot overlay assay. Purified human laminin-5 and the control proteins fibronectin and BSA were blotted onto nitrocellulose. Subsequently, tPA, uPA or plasminogen were incubated in solution with the membranes overnight. The blots were then processed by immunoblotting using antibodies against tPA, plasminogen or uPA. tPA binds to laminin-5 and to fibronectin (Fig. 26). Plasminogen binds only to laminin-5 while uPA binds both BSA and fibronectin (Fig. 26). uPA binds poorly to laminin-5 (Fig. 26).

SUMMARY AND DISCUSSION

Laminin-5 has been reported to function in the nucleation of hemidesmosome assembly and as an adhesive factor that retards cell motility (Baker et al., 1996a; O'Toole et al., 1997). In contrast some authors have provided evidence that laminin-5 enhances cell motility and is expressed at the migrating edges of certain tumor cell populations (Zhang and Kramer, 1996; Pyke et al., 1994; Pyke et al., 1995). The data presented here indicate that post-translational processing of the α3 subunit of laminin-5 may modulate laminin-5 function. In the case of laminin-5 derived from pp126 cells, the α3 subunit is approximately 190 kD, similar to the size of the unprocessed laminin-5 α chain identified by others (Marinkovich et al., 1992; Matsui et al., 1995a). Laminin-5 which contains this unprocessed α3 chain supports cell motility and does not induce hemidesmosome assembly. Upon plasmin-mediated proteolytic cleavage of the α3 subunit to 160 kD, the laminin-5 molecule becomes competent to trigger the assembly of hemidesmosomes, leading to decreased cell motility.

Based on the observation that laminin-5 is expressed at the leading edge of migrating tumor cells one might hypothesize that this laminin-5 includes the 190 kD α3 subunit rather than the 160 kD processed form (Pyke et al., 1994; Pyke et al., 1995). It will be possible to assess this following generation of probes against the approximately 30 kD fragment of the unprocessed α3 subunit which is cleaved off by plasmin. Of course, the actively migrating buds of tumors are likely to be rich in a variety of proteases which could further degrade laminin-5 and impact its function. In this regard, it has been recently shown that the γ2 chain is proteolyzed to a 80 kD species by MMP-2 and that laminin-5 containing the truncated γ2 chain induces cell motility (Giannelli, G., J. Falk-Marzillier, W.G. Stetler-Stevenson, and V. Quaranta. 1996. *Mol. Biol. Cell* 7: 58a).

The specific structural and functional impact of plasmin treament on the α3 chain of pp126 laminin-5 in our in vitro assays led us to investigate the possibility that the plasminogen activator/plasmin system is involved in processing laminin-5 in vivo. Indeed, our data support the hypothesis that codistribution of enzyme (plasmin) and substrate (laminin-5) facilitates modification of laminin-5 structure with a resulting impact on its function. We have shown that plasminogen is associated with laminin-5 matrix in cultured epithelial cells at the morphological level. Furthermore, tPA is also associated only with the matrix of those cells whose laminin-5 contains a processed α3 chain. tPA is not apparently present in matrix of pp126 cells, in which the α3 chain appears in its unprocessed form. Moreover, we show using a dot blot overlay assay that both plasminogen and tPA can bind laminin-5. Intriguingly, we have been able to induce processing of the α3 chain of pp126 cell laminin-5 by simply adding tPA to pp126 cell extracellular matrix. Since tPA is a proteinase with extremely limited substrate specificity, it is unlikely that tPA alone could proteolyze the α3 chain (Stack et al., 1995). Together these data suggest that addition of tPA to the pp126 cells is able to catalyze the conversion of plasminogen to plasmin which thereby can then target the α3 subunit.

The role of plasmin and tPA in generating a truncated laminin-5 α3 subunit in this system has striking parallels to the relationship of plasmin and tPA to laminin-1. For example, extracellular tPA secreted by B16F10 melanoma cells and human colon carcinoma cells induces hydrolysis of laminin-1 in a plasminogen-dependent manner (Stack et al., 1993; Sordat et al., 1995; Tran-Thang et al., 1994). Both tPA and plasminogen exhibit high affinity binding to the α1 subunbit of laminin-1 (Moser et al., 1993). Moreover, full-length laminin-1, as well as a short peptide of the laminin-1 α1 subunit containing the sequence SRARKQAASIKVAV, is able to stimulate the tPA-catalyzed activation of plasmin from plasminogen (Stack and Pizzo, 1993; Stack et al., 1994a). In this regard, the α3 subunit of the laminin-5 isoform contains a similar sequence (IQQARDAASKVAV) just upstream of the putative start of its globular or G-domain. As we have previously shown that the G domain of the laminin-5 α3 chain is essential for nucleating hemidesmosomes (Baker et al., 1996b), tPA/plasmin-mediated cleavage of laminin-5 may generate a truncated, functional G domain that is capable of triggering hemidesmosome assembly. Furthermore, a stimulatory effect of laminin-5 on tPA-induced plasminogen activation, similar to that observed with laminin-1, would support a feedback mechanism, whereby epithelial cells indirectly influence their own behavior by affecting the structure and function of their own matrix molecules in their extracellular environment (Roskelly et al., 1995).

Based on our results, we propose the following model for nucleation of hemidesmosomes in epithelial cells. Laminin-5, containing a 190 kD α3 subunit, is secreted into the extracellular environment by epithelial cells. Plasminogen associates directly with the matrix by binding laminin-5. Only certain epithelial cell types such as MCF-10A secrete tPA which, like plasminogen, also associates with laminin-5. The spatial colocalization of plasminogen and tPA on the laminin-5 molecule allows for efficient production of plasmin from plasminogen, catalyzed by tPA. The newly generated plasmin then initiates cleavage of the α3 subunit of laminin-5. Following cleavage of its α3 subunit, laminin-5 is then able organize the appropriate integrins and other cell surface-associated proteins to nucleate assembly of a hemidesmosome (Baker et al, 1996b). Those cell types such as pp126 cells which do not express tPA in their extruded matrix, are thus incapable of forming hemidesmosomes because the α3 chain of the laminin-5 molecule is incompletely processed.

In summary, we have elucidated a regulatory enzymatic cascade which, in cells secreting the appropriate enzymes, appears to result in proteolysis of laminin-5 and subsequent nucleation of hemidesmosome assembly. Many investigators have recognized the ability of extracellular matrix to alter cellular behavior. Our data, showing the downstream effects of secretion of specific enzymes which bind to and modify laminin-5, indicate that epithelial cells indirectly regulate their own behavior, via changes in the surrounding matrix.

Task 4

In this task we determined that hemidesmosome matrix and integrins appear to play an important role in setting up normal tissue architecture in the breast. This is consistent with our initial hypothesis that disruption of hemidesmosome could lead to development of breast cancer. During the course of these studies we have shown that MCF-10A cells provide an excellent model for studying hemidesmosome assembly as well as the role of hemidesmosomes in normal breast epithelial tissue develpoment. Thus we have used MCF-10A cells rather than a heterogeneous population of primary cells for the subsequent studies.

EXPERIMENTAL RATIONALE

Extracellular matrix plays a crucial role in determining the morphogenesis of a number of epithelial tissue types (Hay, 1993). One of the most dramatic examples of this phenomenon is the regulation of mammary epithelium phenotype by elements of basement membranes derived from the Engelbreth-Holm-Swarm tumor ("matrigel")(Bissell and Ram, 1989; Barcellos-Hoff et al., 1989; Blum et al., 1989; Lin and Bissell, 1993). Indeed, mouse mammary epithelial cells assemble into structures remarkably similar to alveoli of lactating mammary glands and produce milk proteins when maintained in matrigel (reviewed in Lin and Bissell, 1993).

Compared with the rodent system, analyses of morphogenesis of human mammary epithelial cells has progressed more slowly, in part because of difficulties in maintaining cultures of primary human cells. This problem has been partially alleviated by the development of media for the culture of primary human mammary epithelial (HMEC) cells although establishment of primary cultures remains problematic (Stampfer, 1985; Bergstraesser and Weitzman, 1993). One alternative is the use of continuous human mammary epithelial cell lines such as MCF-10A (Soule et al., 1990). Indeed, a model for the study of mammary epithelial cell morphogenesis using MCF-10A cells has recently been described (Howlett et al., 1995).

It has now been shown that laminin-1 is the matrix component of matrigel which regulates morphogenesis as well as milk protein expression of mouse mammary epithelial cells in vitro (Streuli et al., 1995). Furthermore, the domain responsible for such regulation resides in the so-called E3 fragment of laminin-1 and is located towards the carboxy terminus of the α1 subunit of the heterotrimer (Streuli et al., 1995). Laminin-1, via its cell surface receptors, is believed to establish polarity of mammary epithelial cells, a process which is an essential prerequisite to cell differentiation (Streuli et al., 1995). However, following polarization, it is hypothesized that epithelial cells modulate their own microenvironment by producing additional basement membrane components (Bissell and Ram, 1989). The latter could include a number of laminins since laminin-1 is only one of several laminin isoforms which occur in intact basement membranes (Timpl and Brown, 1994). For example, laminin-5 is widely distributed in the basement membranes of epithelial tissues, including the mammary gland, as we show here (Verrando et al., 1987; Rousselle et al., 1991; Carter et al., 1991; Kallunki et al., 1992; Timpl and Brown, 1994). Do these endogenously secreted basement membrane elements play a role in mammary epithelial morphogenesis? To answer this question, we have analyzed the function of

laminin-5 in an in vitro model of mammary epithelial morphogenesis using MCF-10A cells. These cells undergo branching morphogenesis i.e. assemble a highly anastomosed multicellular network, when cultured on matrigel. We show that matrigel-induced differentiation of MCF-10A cells is inhibited by function blocking laminin-5 antibodies as well as antibodies against two distinct laminin-5 receptors. Since laminin-5 is a component of certain cell-matrix junctions called hemidesmosomes and MCF-10A cells assemble hemidesmosomes in vitro, we discuss the possibility of signaling events transduced by these complex morphological entities. This work was published in the Journal of Cell Science and a preprint is included in the Appendix (Stahl et al., 1997).

MATERIALS AND METHODS

Cell Culture

MCF-10A cells were obtained from American Tissue Culture Collection (Rockville, MD) and were maintained in a 1:1 mix of DME and Ham's F12 media supplemented with 5% equine serum, 0.01 mg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone. SCC12 cells were maintained in a serum free growth medium (Medium 154)(Cascade Biologics, Inc., Portland, OR).

For our morphogenesis assays, matrigel was purchased from Collaborative Biomedical Products (Bedford, MA) and was coated onto plastic dishes at approximately 15 mg/ml. The dishes were subsequently incubated at 37^{0}C for 30 minutes prior to addition of cells. In some instances, cells were mixed with liquid matrigel at 4^{0}C. The cell/matrigel mix was then pipetted onto plastic and allowed to gel at 37^{0}C.

Antibodies

GB3, a mouse monoclonal antibody which recognizes the γ chain of human laminin-5, was obtained from Harlan Bioproducts for Science, Inc. (Indianapolis, IN) (Verrando et al., 1987, Matsui et al., 1995). Mouse monoclonal antibody, clone 17, specific for the β chain of laminin-5 was purchased from Transduction Laboratories (Lexington KY). Dr. William Carter, Fred Hutchinson Cancer Research Center generously provided C2-9, a function blocking mouse monoclonal antibody specific for the α3 chain of laminin-5 and P1E1, a non-function blocking antibody which also recognizes the α3 chain of human laminin-5 (Xia et al., 1996). We used P1E1 as a control IgG in some of our antibody inhibition studies. The rabbit serum J17, against BP180 and the mouse monoclonal antibody 10C5, against BP230, have been described elsewhere (Hopkinson et al., 1992; Hopkinson and Jones, 1994). GoH3, a rat monoclonal which recognizes the α6 integrin subunit, was purchased from Immunotech (Westbrook, ME). P1B5 and 3E1, mouse monoclonal antibodies which recognize the α3 integrin and β4 integrin subunits respectively, were purchased from Gibco BRL (Gaithersburg, MD). Rabbit sera 6945 and 6845, against β4 integrin and the "light" chain of the α6 integrin subunit respectively, were kindly provided by Dr. Vito Quaranta, Scripps Institute (Tamura et al., 1990) .

Immunofluorescence

MCF-10A cells, maintained on glass coverslips, were either permeabilized in acetone at -20^{0}C for 2 minutes and air dried thoroughly, or, for integrin localization, were first fixed for 5 minutes in 3.7% formaldehyde, washed thoroughly in PBS, and then permeabilized in acetone at -20^{0}C for 2 minutes prior to air drying. Cells maintained in matrigel were prepared for immunofluorescence analyses by first fixing them for 10 minutes in 3.7% formaldehyde. After washing thoroughly in PBS, they were permeabilized with 0.5% Triton-X 100 in PBS at 4^{0}C for 10 minutes and then washed once again in PBS. Preparations were incubated with primary antibody diluted in PBS at 37^{0}C in a humid chamber for 1 hour, washed 3 times in PBS, and

incubated with an appropriate fluorochrome conjugated secondary antibody for a further 1 hour at 37^{0}C.

For frozen tissue sections, normal human breast tissue from a reduction mammoplasty was received from the Cooperative Human Tissue Network (Columbus, OH). Tissue was snap frozen in liquid nitrogen and embedded in Tissue-Tek OCT compound (Miles Laboratory, Elkhart, IN). 10 μm thick sections of the frozen tissue were prepared and mounted on poly-L-lysine coated microscope slides. Sections were fixed for 5 minutes in -20C acetone, air-dried thoroughly, and stained for immunofluorescence as above.

Fluorescence specimens were visualized using a Zeiss LSM10 laser scanning confocal microscope (Zeiss Inc., Thornwood, NY). Images were stored on Sony optical discs and printed on a Tektronix printer (Tektronix, Wilsonville, OR).

Protein Preparations, SDS-PAGE and Western Immunoblotting

Confluent cell cultures were solubilized in sample buffer consisting of 8 M urea, 1% sodium dodecyl sulfate (SDS) in 10 mM Tris-HCl, pH 6.8 and 15% β-mercaptoethanol. DNA was sheared by sonication using a 50 watt Ultrasonic Processor (Vibracell Sonics and Materials Inc., Danbury, CT). Matrix of MCF-10A cells was prepared according to Gospodarowicz (1984) and solubilized in sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and processed for immunoblotting according to Zackroff et al. (1984).

Immunoprecipitation

Subconfluent dishes of MCF-10A cells were radiolabeled overnight with 50 μCi/ml of ^{35}S-PRO-MIX cell label (Amersham Corp., Arlington Heights, IL). Conditioned medium of the labeled MCF-10A cells was collected and then pre-cleared by incubation with protein G sepharose beads (Gibco BRL, Gaithersburg, MD) for one hour at 4^{0}C. After centrifugation, monoclonal antibodies were added to the supernatant and the mix was then incubated for 1 hour at 4^{0}C. Protein G sepharose beads were added and the tubes incubated for an additional hour at 4^{0}C. Beads were collected by centrifugation and washed 5 times in TBS (10 mM Tris-HCl, pH 7.4, 145 mM NaCl and 1mM PMSF) containing 1% Triton X-100. Proteins eluted from the beads in sample buffer were processed for SDS-PAGE/autoradiography as well as immunoblotting.

Electron Microscopy

Cells maintained on tissue culture plastic or in matrigel were fixed for a minimum of 30 minutes in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After being washed three times in 0.1 M sodium cacodylate buffer, cells were post-fixed in 1% OsO_4 containing 0.8% potassium ferricyanide. Preparations were subsequently stained with uranyl acetate, dehydrated in ethanol, and embedded in Epon-Araldite resin (Tousimis Corp., Rockville, MD). Thin sections of embedded material were stained with lead nitrate and sodium citrate and viewed at 60 kV in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA).

RESULTS

MCF-10A Cells Express Laminin-5 as well as Hemidesmosomal Proteins

MCF-10A cells were maintained on glass coverslips for 24 hours and then processed for immunofluorescence microscopy using monoclonal antibodies against laminin-5. The latter stain in a leopard spot pattern along sites of cell-substrate association as determined by confocal laser scan microscopy (Fig. 27A). Laminin-5 antibody reactivity also occurs along areas of the glass coverslip where there are no apparent cells, suggesting that the MCF-10A cells leave behind "trails" of laminin-5 as they migrate over their substrate. Since MCF-10A cells are derived from human mammary glands, we also determined whether laminin-5 is a component of breast epithelial basement membranes. Indeed, basement membranes encircling groups of breast epithelial cells show strong reactivity with laminin-5 antibodies in cryosections of mammary tissue material (Fig. 27C).

In addition to laminin-5, MCF-10A cells, processed for indirect immunofluorescence microscopy, are recognized by antibodies against major components of hemidesmosomes including both bullous pemphigoid antigens (BP180, BP230) as well as the β4 and α6 integrin subunits (Jones et al., 1994; Green and Jones, 1996)(Fig. 28). All of these antibodies generate similar leopard spot staining patterns along the basal aspect of the adherent cells (Fig. 28). This pattern is comparable to that generated by laminin-5 antibodies (Fig. 27A). However, unlike laminin-5, there is an absence of hemidesmosome protein in areas of the glass coverslips devoid of cells (Fig. 28).

Electron microscopic analyses of MCF-10A cells reveals that they assemble hemidesmosome-like structures where they abut their substrates (Fig. 29). These structures possess all of the morphological features of hemidesmosomes observed in mammary epithelial cells in situ i.e. they have triangular shaped, trilayered cytoplasmic plaques (Fig. 29; Jones et al., 1994; Bergstraesser et al., 1995).

To confirm that MCF-10A cells express hemidesmosome components, we have analyzed cell extracts by immunoblotting using antibodies directed against BP180 and BP230, and antisera against β4 integrin and the "light" chain of α6 integrin (Fig. 30A). These antibodies recognize species of 180, 230, 200 and 30kD respectively (Fig. 30A, lanes 1,3,5 and 7). Furthermore, the MCF-10A hemidesmosomal proteins co-migrate with their epidermal equivalents present in extracts of SCC12 cells (Fig. 30A, lanes 2,4,6 and 8).

MCF-10A Cells Produce a Laminin-5 Rich Matrix and Secrete Soluble Laminin-5

We have analyzed both the matrix deposited onto substrates by MCF-10A cells as well as MCF-10A conditioned medium for the presence of laminin-5 using a combination of immunoblotting and immunoprecipitation. MCF-10A matrix was prepared according to the procedure of Gospodarowicz (1984). This matrix contains four prominent polypeptides of 155, 135, 100 and 80kD and is rich in subunits of laminin-5 as shown by immunoblotting using a monoclonal antibody which recognizes the β2 135kD laminin-5 subunit (Fig. 30B). In addition, the 155, 135 and

100kD species present in MCF-10A matrix co-migrate with the major polypeptides immunoprecipitated from MCF-10A conditioned medium by two laminin-5 monoclonal antibodies (GB3 and C2-9)(Fig. 30C, lanes 1 and 3). The 135kD polypeptides immunopreciptated from MCF-10A conditioned medium by both these anti-laminin-5 monoclonal antibodies are recognized by the β2 chain antibody in immunoblots (Fig. 30C, lanes 2 and 4).

MCF-10A Cells Undergo Branching Morphogenesis when Plated on Matrigel

When MCF-10A cells are embedded into liquid matrigel, which is then allowed to gel, they remain as discrete cellular aggregates ("acini") for 7 days or more regardless of cell concentration (Howlett et al., 1995). In contrast, MCF-10A cells form an interconnected set of tube-like structures, one day after being plated at a concentration of $2.5x10^4$ cells/cm^2 on top of matrigel (Fig. 31A). These are similar to the networks of HMECs observed in matrigel and collagen I gels (Bergstraesser and Weitzman, 1996; Berdichevsky et al., 1994).

The ability of MCF-10A cell to assemble into tube-like arrays is cell concentration dependent. At cell concentrations of $1.25x10^4/cm^2$ or below the MCF-10A cells remain as small aggregates on the matrigel (Fig. 31B). Indeed, they remain in similar aggregates even at 7 days following plating (result not shown).

The tube-like multicellular aggregates of MCF-10A cells in matrigel were processed for confocal immunofluorescence microscopy using antibodies against laminin-5, α6 integrin and α3 integrin (Fig. 32). Both laminin-5 and α6 integrin are concentrated along the edges of the MCF-10A tubes where the cells abut matrigel (Fig. 32A,B). α3 integrin is localized at the latter sites although it is also present at areas of cell-cell contact (Fig. 32C). An IgG control fails to stain the cell population in Fig. 32D.

Antibody Inhibition of MCF-10A Morphogenesis

We next used an immunological approach to assess the potential role of laminin-5 and its receptors (the integrins α6β4 and α3β1) in matrigel induced branching morphogenesis of MCF-10A cells. For these studies MCF-10A cells were incubated for 15 minutes at 37^0C in medium containing either control IgG (50 μg/ml) or in function blocking antibodies directed against α6 integrin (GoH3 at 50 μg/ml), α3 integrin (P1B5 diluted 1:20) and laminin-5 (C2-9 diluted 1:5)(Fig. 33). The cells in the antibody containing medium were plated onto matrigel coated surfaces at $2.5x10^4/cm^2$. After 24 hours the cells incubated in control IgG had formed long interconnected tubes whereas there was an obvious inhibition of branching morphogenesis in cultures which had been incubated in the α3 and α6 integrin antibodies as well as those cells incubated with the laminin-5 antibodies (Fig. 33).

We also fixed and processed the antibody treated cells for electron microscopy. We analzyed at least twenty MCF-10A cells in contact with matrigel under each experimental condition (Fig. 34). MCF-10A cells plated onto matrigel in the presence of control IgG assemble hemidesmosomes at sites of cell-matrigel association (Fig. 34A). The latter appear as electron dense structures with

extracellular sub-basal dense plates which indicate formation of "mature" hemidesmosomes (Fig. 34A, inset). In contrast, no hemidesmosome were observed along regions of cell-matrigel interaction in cultures incubated in function blocking α3 integrin, α6 integrin and laminin-5 antibodies (Fig. 34B-D).

SUMMARY AND DISCUSSION

In this study we have shown that MCF-10A cells, an immortalized mammary epithelial cell line, like HMECs, derived from reduction mammoplasties, undergo a branching morphogenesis when maintained on top of matrigel (Bergstraesser and Weitzman, 1996). This phenomenon is highly dependent on cell concentration. We have never observed the formation of tubular arrays when MCF-10A cells are plated onto matrigel at concentrations below $1.25x10^4$cells/cm^2. Just a two fold increase in this cell number is enough to trigger a matrigel induced branching morphogenesis of the MCF-10A cells. Indeed, we find it remarkable that within 1 day of plating onto matrigel, MCF-10A cells assemble into an anastomosing network, organized into a branching pattern much like that seen in vivo in postpubertal mammary glands (Daniel and Silberstein, 1987). This type of pattern has been observed by Berdichevsky et al. (1994) when the human mammary cell line HB-2 is maintained in collagen type I gels.

HMECs assemble hemidesmosomes in vivo (Watson et al., 1988). In vitro they are also capable of forming hemidesmosomes, although this generally takes up to 14 days following plating on tissue culture substrates (Bergstraesser et al., 1995). Like HMECs in vivo, MCF-10A cells express the major components of hemidesmosomes as determined by immunofluorescence, immunoblotting and immunoprecipitation. Moreover, MCF-10A cells readily assemble hemidesmosome-like structures within 24 hr after plating onto uncoated glass coverslips i.e. much faster than their normal counterparts. The speed of hemidesmosome appearance in MCF-10A cells was the more surprising since earlier work had suggested that MCF-10A cells were unable to assemble bona fide hemidesmosomes in vitro (Tait et al., 1990).

When maintained on matrigel, MCF-10A cells assemble hemidesmosomes at sites of cell-matrigel interaction. Consistent with this, a hemidesmosome associated matrix component and its receptor, namely laminin-5 and $\alpha6\beta4$ integrin, are distributed at sites of MCF-10A cell-matrigel interaction. Such observations triggered our interest in the potential role of hemidesmosome components in branching morphogenesis of MCF-10A cells. Since it is already established that laminin-5 and $\alpha6\beta4$ integrin heterodimer are essential for hemidesmosome assembly, we have been able to assay the role of hemidesmosomes in branching morphogenesis of MCF-10A cells by using antibodies which inhibit both the activities of laminin-5 and $\alpha6\beta4$ integrin (Jones et al., 1991; Kurpakus et al., 1991; Spinardi et al., 1995; van der Neut et al., 1996; Georges-Labouesse et al., 1996; Baker et al., 1996b).

Function blocking antibodies directed against laminin-5 not only prevent hemidesmosome assembly in MCF-10A cells maintained on matrigel but also significantly inhibit branching morphogenesis. Similarly, antibody GoH3, which blocks $\alpha6$ integrin function, inhibits both hemidesmosome formation and MCF-10A morphogenesis. Since the $\alpha6$ integrin subunit is known to preferentially bind $\beta4$ integrin in cells which coexpress both of its $\beta1$ and $\beta4$ integrin partners, as is the case in MCF-10A cells, the inhibitory effects of GoH3 antibodies on MCF-10A cells

likely impact the function of the hemidesmosome-associated α6β4 integrin heterodimer (Giancotti et al., 1992; Baker and Jones, unpublished observations).

Indeed, we assume that matrigel, or more specifically its laminin-1 component, provides an initial framework for MCF-10A attachment and triggers a series of morphogenetic events (Streuli et al., 1995). This includes secretion of laminin-5 which then induces the MCF-10A cells to nucleate the assembly of their own hemidesmosomes, a process requiring laminin-5/α6β4 integrin interaction. We suggest that the formation of the latter complex is necessary to complete branching morphogenesis.

The idea that hemidesmosomes may be involved in morphogenetic events is supported indirectly by recent reports which indicate that hemidesmosomes are sites of signal transduction (Maniero et al., 1995; Maniero et al., 1996). For example, the β4 subunit of the α6β4 hemidesmosome associated integrin possesses an unusually long cytoplasmic tail which is associated with one or more protein kinases (Tamura et al., 1990; Maniero et al., 1995). The latter are believed to be involved in a matrix induced cascade of phosphorylation events resulting in phosphorylation not only of the β4 integrin subunit but also of a recently identified protein of 80kD (Xia et al., 1996; Maniero et al., 1995).

Laminin-5 and α6 antibodies are not exclusive in their abilities to block morphogenesis of MCF-10A cells in matrigel. A function perturbing α3 integrin antibody, P1B5, is also capable of inhibiting matrigel induced branching morphogenesis of MCF-10A cells. The α3β1 integrin heterodimer is not a component of the hemidesmosome but, like α6β4 integrin is a receptor for laminin-5 (Carter et al., 1990; Carter et al., 1991). In in vitro assays, it has been shown that cell interaction with laminin-5 is initiated by the α3β1 integrin heterodimer (Carter et al., 1991). Subsequently laminin-5 appears to "switch" receptors and binds to the α6β4 integrin as a prelude to hemidesmosome assembly (Carter et al., 1990; Carter et al., 1991; Spinardi et al., 1995; Xia et al., 1996). Thus one explanation for the morphogenetic impact of the α3 integrin blocking antibody is that P1B5 inhibits the interaction of cells with their own laminin-5. However, we cannot discount that α3 integrin is involved in cell binding to the laminin-1 component of matrigel (Streuli et al., 1995). Of course, P1B5 may inhibit both laminin-1 and laminin-5 interactions of the MCF-10A cells.

In summary, we have identified a model system and a continuous cell line, MCF-10A, for the study of the role of hemidesmosome matrix and integrin components in tissue morphogenesis. In this model, matrigel provides a three dimensional environment which triggers a series of cellular morphogenetic events, involving the assembly of hemidesmosomes and expression of hemidesmosome matrix and integrin components, in MCF-10A cells. Indeed, it is becoming clear that the hemidesmosome is not simply a spot weld to tether cells to connective tissue but, through the functional properties of its components, the hemidesmosome can have profound impact on the differentiation and organization of epithelia at the tissue level.

Figure Legends

Figure 1. The presence of hemidesmosomes in normal breast epithelium by electron microscopy.
A. A normal duct in cross section. B. Higher magnification of box in "A" shows hemidesmosomes (arrows) at the bases of ductal epithelial cells. C. Higher magnification of a hemidesmosome showing intermediate filaments (IF, curved arrows), anchoring filaments (arrow) and anchoring fibrils (arrowheads along the length). LL=lamina lucida, LD=lamina densa. D. A luminal cell reaching from lumen to basement membrane containing basal hemidesmosomes (arrows). E. Higher magnification of the box in "D" showing hemidesmosomes (arrows). L=lumen, E=epithelial cell, F=fibroblast, ECM=extracellular matrix.

Figure 2. Presence of hemidesmosomes in breast carcinoma in situ by electron microscopy.
A. An intraductal carcinoma showing many layers of cells within a duct with very little lumen (L) but surrounded by a basement membrane (BM). B. Higher magnification of the box in "A" shows multiple hemidesmosomes (arrows). C=carcinoma cell, ECM=extracellular matrix, F=fibroblast.

Figure 3. Absence of hemidesmosomes in invasive breast carcinoma.
A. A group of invasive cells is pictured. Note the absence of a basement membrane at the epithelial:extracellular matrix border (curved arrows), presence of desmosomes (D, straight arrows). A cell, apparently at the invasive front, is surrounded by extracellular matrix on three sides (box). C=carcinoma cell, ECM=extracellular matrix, F=fibroblast, P=pseudolumen. B. Higher magnification of the box in "A" shows two invasive cells (1 and 2) abutting the extracellular matrix (ECM) without any hemidesmosomes. C. Higher magnification of box in "B" shows cells (1 and 2) with cell membranes in direct contact with collagen (arrows) without any intervening basement membrane or hemidesmosomes.

Figure 4. Expression of the anchoring fibril protein, collagen VII in normal duct and carcinoma in situ, but not invasive carcinoma by immunofluorescence.
A-C. A normal duct in cross section (A) has a basement membrane as determined by laminin staining (B) and expresses basal collagen VII (C). D-F. An intraductal carcinoma (D) has a basement membrane around the entire group of cells (E) and exhibits collagen VII staining (F) only at the basement membrane (arrowheads) whereas cells piled up within the duct do not exhibit collagen VII staining (arrow, compare with D, arrow). G-I. Invasive carcinoma (G), outlined by arrowheads, does not have a basement membrane (H) or express collagen VII (I). Ln=laminin, C VII=collagen VII, L=lumen.

Figure 5. Expression of the M_r 180 bullous pemphigoid antigen and hemidesmosome protein by immunofluorescence.
A. Normal ducts in cross section show basal M_r 180 staining. B. In regions of intraductal carcinoma, staining for the M_r 180 protein outlines ducts from most patients. C. In one

patient, no M_r 180 protein staining is seen even in regions of carcinoma in situ. D, E. Phase contrast photographs of "B" and "C" respectively.

Figure 6. Expression of the M_r 200 hemidesmosome protein does not follow the basement membrane by immunofluorescence.
A. Negative control for immunofluorescent staining. B, C. A normal duct in cross section has a basement membrane illustrated by collagen IV staining (B) and expresses basolateral M_r 200 protein (C). D-F. An intraductal carcinoma in longitudinal section (D) has a basement membrane (E, arrowheads), but no M_r 200 protein staining (F). G-I. Invasive carcinoma (G) exhibits intracellular basement membrane protein staining (H), and faint M_r 200 protein staining surrounding each cell (I). C IV=collagen IV.

Figure 7. Electron microscopy of normal and malignant human mammary epithelial cells in culture.
A. A normal cell has basal hemidesmosomes (arrows). B. A malignant cell has no hemidesmosomes. C. Higher magnification of box in "A". D. Higher magnification of box in "B".

Figure 8. Delayed expression of the anchoring fibril protein, collagen VII, in cultured malignant human mammary epithelial cells by immunofluorescence.
A. Normal cultured human mammary epithelial cells at one day show an intracellular staining pattern for collagen VII. B. Malignant human mammary epithelial cells do not express collagen VII at one day. C. By seven days normal cells stain for collagen VII at the base of cells in a secreted pattern. D. At seven days malignant cells show an intracellular staining pattern for collagen VII.

Figure 9. Aberrant expression of the M_r 200 hemidesmosome protein in cultured malignant human mammary epithelial cells by immunofluorescence.
A. Normal cells express the M_r 200 protein in rows of basal tick mark-shaped plaques.
B. Malignant cells show a dotted intracellular M_r 200 protein staining pattern.

Figure 10. Identical expression of the M_r 180 hemidesmosome protein in normal and malignant cultured human mammary epithelial cells.
Both normal (A) and malignant (B) cells express the M_r 180 protein in rows of basal tick mark-shaped plaques.

Figure 11. Electron microscopy of normal HMEC cultured on Matrigel.
Cells were grown on Matrigel BM-like substance for 20 days. During this time they underwent differentiation into three-dimensional duct-like structures and assembled hemidesmosomes (arrows) which were associated with intermediate filaments (arrowheads).

Figure 12. Electron microscopy of normal HMEC cultured on 804G matrix.
A. HMEC grown on glass coverslips for 24 hours have no hemidesmosomes.

B. HMEC grown on laminin-rich 804G matrix for 24 hours contain hemidesmosomes (arrowheads).

Figure 13. FG cells were plated into control medium and 24 hr later processed for immunofluorescence using monoclonal antibody GoH3 to the α6 integrin subunit (A). Confocal microscopy shows that α6 staining consists of patches and streaks in a plane of focus located at the basal aspect of the cell. B, phase contrast image. Bar, 25 μm.

Figure 14. A. Approximately 10 μg of extracts of FG cells maintained for 24 hr in their normal medium (lane 1) or for 6 hr in laminin-5 rich medium (lane 2) were prepared for SDS-PAGE on 12% gels. Separated polypeptides were then transferred to nitrocellulose and subsequently processed for immunoblotting using 4E9G8 antibody. The latter shows reactivity with a 25 kD protein only in lane 2. Molecular weight standards indicated by dashes are 200, 97, 66, 45, 31 and 21 kD.
B. The α6A integrin cytoplasmic non-phospho- and phospho-peptides at 1 mg/ml or 0.1mg/ml (from left to right) were spotted onto PVDF membrane. The PVDF pieces were then processed for immunoblotting either using 4E9G8 monoclonal antibody or serum 6844. Both peptides are recognized by antibodies in the 6844 serum whereas 4E9G8 fails to recognize the phospho-peptide.
C. FG cells, maintained in the absence (-LN5) or presence (+LN5) of rat laminin-5, were labeled with ^{32}P. The α6 integrin subunit was then immunprecipitated from the labeled cells using monoclonal antibody GoH3. Equal amounts of precipitated α6 integrin were subjected to SDS-PAGE and were either transferred to nitrocellulose and processed for immunoblotting using the α6A integrin serum, 6844, or visualized by autoradiography. The reactivity of the 6844 antibodies on the immunoblot indicates that equal amounts of α6A integrin from the FG cells have been loaded onto the two lanes. This was confirmed by scanning densitometry. In contrast, the autoradiograph shows an apparent 37% decrease in the level of phosphorylation of α6A in the GoH3 antibody precipitates derived from FG cells maintained in the presence of laminin-5 (arrow). The asterisk marks cross reactivity of the blotting secondary antibody with the precipitated IgG. Molecular weight standards are (from top to bottom) 97, 66, and 45kD

Figure 15. At 24 hr following plating, FG cells maintained in their normal medium were processed for indirect immunofluorescence using the α6 monoclonal antibody 4E9G8 (A). The antibody generates no obvious staining pattern (the focal plane shown is close to the substratum attached surface of the cells). In contrast, in FG cells maintained for 6 hr in laminin-5 rich medium, antibody 4E9G8 generates staining in circles (C). These circles co-localize with circles stained by GoH3 antibodies (open arrow, E and F). However, it should be noted that GoH3 staining is more extensive than that of 4E9G8 antibodies (closed arrows, E and F). B, D and G show phase contrast images of the cells. Bar in B, 10 μm; bar in G, 25 μm.

Figure 16. Cryosections of normal (A-F) and invasive tumor-containing (G-L) breast tissues were processed for double label immunofluorescence microscopy using GoH3 antibodies (A,D,G and J) in combination with either 4E9G8 (B and H) or GB3 (E and K) antibodies. Sections shown in A-C and D-F, as well as the sections shown in G-I and J-L, were collected consecutively from the microtome. This allowed us to photograph the same islands of normal (and tumor cells) in the distinct double labels. Note that in A,B and D,E all of the antibodies generate similar staining, concentrated at regions of epithelial cell-matrix interaction. In G and J, GoH3 antibodies recognize an island of tumor cells in the absence of corresponding 4E9G8 (H) and GB3 (K) staining. C,F,I and L, phase images; bar, 25 μm.

Figure 17. 804G cells or MCF-10A cells were plated onto tissue culture plastic, or surfaces coated with 5 $\mu g/cm^2$ rat tail collagen (RTC), 25 μg/ml fibronectin (FN), 25 μg/ml laminin-1 (Ln 1) and either 1 μg/ml human or 2 μg/ml rat laminin-5 (hLn 5 or rLn 5) for 48 hours. As indicated, the 804G and MCF-10A cells were maintained in medium supplemented with either 50 μg/ml IgG control antibody or 50 μg/ml laminin-5 function inhibiting antibodies CM6 and P3H9-2. In the case of MCF-10A cells, one batch of P3H9-2 antibody treated cells were also incubated in the β1 integrin activating antibody TS2/16.2.1. At 48 hours the cells were trypsinized and counted. The "Average index" indicates the fold increase in cell number (the number of cells collected after 48 hours divided by the number of cells originally plated [2 X 10^4 cells/well of a 24 well plate]). The standard deviation was determined from the data derived from thouree trials.

Figure 18. Phase contrast images of 804G (A-C) and MCF10A cells (D-F) are shown at 24 hours following plating into control medium (A,D), medium supplemented with 50 μg/ml of the appropriate laminin-5 function inhibiting antibodies CM6 and P3H9-2 (B,E respectively) or medium containing 50 μg/ml of control IgG (C,F). Note that in all cases the cells have attached to and spread onto their substrate. Bar, 500 μm.

Figure 19. 804G cells were maintained in the presence of CM6 antibody (A,B) or control IgG (C,D). After 24 hours the cells were extracted in detergent and then fixed and stained using a BM28 antibody preparation (A,C) and DAPI (B,D). The same range of BM28 staining patterns is observed in the nuclei of the 804G cells in both A and C. Bar, 10 μm.

Figure 20. Rat (lane 1) and human (lane 2) laminin-5 prepared from the conditioned medium of 804G and MCF-10A cells respectively were subjected to SDS-PAGE on 6% resolving gels. The bracketed polypeptides in lane 1 and 2 are (from top to bottom) the α3, γ2 and β3 subunits of laminin-5. The lower species are proteolytic fragments of these chains. Molecular weight standards are indicated to the right.

Figure 21. The laminin-5 subunit compositions of the extracellular matrices (ECM) of MCF-10A, 804G, pp126, SCC12 and NHEK cells are shown (lanes 1-6). Approximately 10μg of matrix protein was run on each lane of a 6% gel, transferred to nitrocellulose and then processed for immunoblotting using laminin-5 subunit-specific antibody preparations. In a, the laminin-5 β3 chain was identified using the monoclonal antibody clone 17. Clone 17 antibody recognizes a protein of 145 kD in all of the human matrices but shows no reactivity with 804G cell matrix (compare lane 2 with lanes 1,3,5 and 6 in a). In b, antiserum J20, against the laminin-5 γ2 chain, recognizes 155 kD polypeptides in matrix preparations derived from MCF-10A, pp126 and NHEK cells (lanes 1,3 and 6) as well as a 105 kD species in the matrices of MCF-10A, 804G, SCC12 and NHEK cells (lanes 1,2,5 and 6). In c, the laminin-5 α3 chain is identified using the mouse monoclonal antibody 10B5, which recognizes a polypeptide migrating at 160 kD in 804G and MCF-10A cell matrix (lanes 1 and 2) and a protein of 190 kD in the matrices of pp126, NHEK and SCC12 cells (lanes 3,5 and 6).

Approximately 50 μg of the matrix of pp126 cells was treated for 90 min with a 1 ml PBS solution containing plasmin (+Pm) at a concentration of 1μg/ml and then the treated matrix preparation was probed with the β3, γ2 and α3 subunit specific antibodies (lane 4 in each blot). The mobility of the β3 and γ2 subunits are unaffected by such treatment (compare lanes 3 and 4 in a and b) whereas the α3 subunit migrates at 160 kD, compared with 190 kD in the untreated matrix (compare lanes 3 and 4 in c). Molecular weight standards are indicated to the left.

Figure 22. SCC12 cells were plated onto pp126 ECM, pp126 ECM which had been treated with plasmin (Pm)(1 μg/ml for 90 min), MCF-10A ECM, affinity purified pp126 laminin-5 and affinity purified pp126 laminin-5 treated with plasmin (Pm)(1 μg/ml for 90 min). After 1 hr the motility of the SCC12 cells was assayed by video microscopy and quantitated as the average of the total displacements of each cell over a two hour period. Approximately thirty cells were evaluated for each trial. Bars represent the standard deviations. Note that SCC12 cells show similar motility on pp126 ECM and affinity purified pp126 laminin-5. They show less motility on plasmin treated pp126 matrix, plasmin treated affinity purified pp126 laminin-5 and MCF-10A ECM. Error bars indicate standard deviations.

Figure 23. Laminin-5-rich pp126 cell matrices were prepared according to Langhofer et al. (1993). SCC12 cells, maintained for 36 hours on either untreated (a) or plasmin-treated (1 μg/ml for 90min) (b) pp126 matrix, were processed for electron microscopy. SCC12 cells on untreated pp126 matrix assemble no obvious hemidesmosomes along regions of cell-matrix interaction (a). In contrast, in b, several hemidesmosomes are observed at sites of cell-matrix association (one is indicated by the arrowhead). These are associated with intermediate filaments (arrow). The inset shows a higher power view of the hemidesmosome marked by the arrowhead in b. It has a triangular electron dense cytoplasmic plaque, sub-basal dense plate (arrow), and anchoring filaments. Bar in a, 300nm; bar in inset, 85nm.

Figure 24. MCF-10A cells (a-c, g-i) and pp126 cells (d-f, j-l) were processed for indirect double-label immunofluorescence microscopy using antibodies against laminin-5 (a,d,g,j) in combination with either an antibody against plasminogen (b,e) or tPA (h,k). Note that there is colocalization of plasminogen and laminin-5 staining patterns in a and b as well as d and e. In g and h, tPA shows codistribution with laminin-5. In k tPA localizes to cell bodies but does not co-localize with laminin-5 in j. c,f,i and l are phase images. Bar, 25μm.

Figure 25. Approximately 10 μg of untreated MCF-10A cell matrix (lane 1), untreated pp126 cell matrix (lane 2), or pp126 cell matrix treated for 16 hrs with tPA (approximately 50 μg of matrix incubated for 16 hrs with 1 ml of tPA at a concentration of either 5 or 10μg/ml)(lanes 3,4) were subjected to SDS-PAGE on 6% gels, transferred to nitrocellulose and then processed for immunoblotting using the laminin-5 α3 subunit antibody 10B5. In untreated pp126 cell matrix the α3 subunit shows a molecular weight of 190 kD (lane 2)(see also Fig. 1). The α3 chain from tPA-treated pp126 matrices comigrates with the α3 chain of MCF-10A matrix at 160 kD (lanes 1, 3 and 4). The asterisk indicates residual 190 kD polypeptide in the 5μg/ml tPA-treated pp126 matrix (lane 3). Molecular weight standards are shown to the left.

Figure 26. Approximately 1 ng of laminin-5 (LN-5), fibronectin (FN) and bovine serum albumin (BSA) were dotted onto nitrocellulose (indicated to the left). Following blocking, the membranes were incubated with 20 μg/ml of tPA, plasminogen (Pg) or uPA overnight at 4^0C (indicated along the top). Bound protein was then detected with the appropriate antibody. Note that tPA binds both fibronectin and laminin-5 while plasminogen binds only laminin-5. uPA binds both BSA and fibronectin and also shows some binding to laminin-5.

Figure 27. Laminin-5 is expressed by MCF-10A cells and in human breast tissue. MCF-10A cells were cultured on glass coverslips and processed for indirect immunofluorescence microscopy using the laminin-5 monoclonal antibody (GB3)(A). The cells were viewed by confocal microscopy, the plane of focus being close to the cell-substrate interface. The laminin-5 antibodies stain in a typical leopard spot pattern. The GB3 antibodies also stain areas where there are no apparent cells (arrow). (C) A cryosection of human breast tissue from a reduction mammoplasty was processed for immunofluorescence with GB3 antibodies. These stain the basement membrane zones of islands of epithelial cells. Panels B and D show phase contrast images. Bars, 10μm.

Figure 28. Hemidesmosomal proteins are expressed by MCF-10A cells as shown by indirect immunofluorescence. MCF-10A cells, maintained on glass coverslips were processed for indirect immunofluorescence microscopy using antibodies specific for

BP180 (J17)(A), BP230 (10C5)(C), β4 integrin (3E1)(E), and α6 integrin (GoH3)(G). In all cases the antibodies generate a patchy, leopard spot stain along the region of cell-coverslip interaction. B,D,F and H show phase contrast images of the cells. Bar, 10μm.

Figure 29. MCF-10A cells assemble hemidesmosomes when maintained in vitro. This electron micrograph shows a cross section of MCF-10A cells. Arrows indicate numerous electron dense hemidesmosome structures. These possess tripartite cytoplasmic plaques (inset, arrow). Bar, 500nm. Bar in inset, 250nm.

Figure 30. A. Hemidesmosomal proteins are expressed by MCF-10A cells as shown by immunoblotting. MCF-10A cell extracts (lanes 1,3,5 and 7) and extracts of SCC12 cells, a keratinocyte line, (lanes 2,4,6 and 8) were separated by SDS-PAGE on either 6% (lanes 1-6) or 15% (lanes 7,8) polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antibodies against BP180 (J17, lanes 1,2), BP230 (10C5, lanes 3,4), β4 integrin (6945, lanes 5,6), or the "light" chain of α6 integrin (6845, lanes 7,8).
B. MCF-10A cells deposit laminin-5 on their substrate. MCF-10A matrix was collected according to Gospodarowicz (1984), processed for SDS-PAGE on a 6% gel, and either silver stained (lane 1) or transferred to nitrocellulose and immunoblotted with a monoclonal antibody (clone 17) against the β chain of laminin-5 (lane 2). In the silver stained preparation, there are prominent polypeptides at 150, 135 and 100 kD representing the α, β and the γ chains of laminin-5 (lane 1). The 135kD protein in this preparation is recognized by the clone 17 antibody (lane 2).
C. MCF-10A secrete laminin-5 into their medium. The medium conditioned by radio-labeled MCF-10A cells was processed for immunoprecipitation using two monoclonal laminin-5 antibodies (GB3, lanes 1,2; C2-9, lanes 3,4). The immunoprecipitated proteins were analyzed by SDS-PAGE/autoradiography (lanes 1 and 3) or prepared for immunoblotting using clone 17 monoclonal antibody against the β chain of laminin-5 (lanes 2 and 4). The laminin-5 antibodies precipitate three major polypeptides of 150, 135 and 100kD (lanes 1,3). The 135kD protein is recognized by the clone 17 antibody (lanes 2,4). Note that there is some breakdown of the laminin-5 in the C2-9 antibody precipitate (lane 2). This may explain the ladder of proteins recognized by the clone 17 antibody in lane 4. The low molecular weight reactive species in lanes 2 and 4 are due to cross reactivity of the secondary antibody anti-mouse IgG with the immunprecipitated mouse IgG.
Dashed lines on the left side of panels A, B, and C indicate weight standards of 200, 116. 97.4, and 66 kD. Dashed lines on the right side of A indicate standards of 66, 45, 31, 21.5 and 14.5 kD. Each lane of the gels was loaded with approximately 10 μg of protein.

Figure 31. MCF-10A cells undergo branching morphogenesis on matrigel in a cell concentration dependent manner. $2.5x10^4/cm^2$ (A) and $1.25x10^4/cm^2$ (B) MCF-10A cells were plated onto matrigel which had been used to coat 35mm dishes. At 24

hours following plating, the cells in A have undergone a branching morphogenesis while the cells in B appear in small aggregates. Bar, 500μm.

Figure 32. Laminin-5 and its receptors are expressed by MCF-10A cells undergoing morphogenesis on matrigel. MCF-10A cells maintained in matrigel for 24 hours were processed for indirect confocal immunofluorescence with monoclonal antibodies recognizing laminin-5 (GB3, A), α6 integrin (GoH3, B), α3 integrin (P1B5, C), or an IgG control (D). Note that the antibodies in A,B and C show staining along regions of cell-matrigel interaction. The insert in (C) is a higher magnification of the boxed area and reveals that α3 integrin occurs at sites of cell-cell as well as cell-matrigel interaction. Bar in A, 100μm; Bar in C, 25μm.

Figure 33. Branching morphogenesis of MCF-10A cells on matrigel is inhibited by antibodies against laminin-5, α6 integrin and α3 integrin. MCF-10A cells were plated onto matrigel in the presence of control IgG (A), or antibodies which block the function of α6 integrin (GoH3, B), α3 integrin (P1B5, C), or laminin-5 (C2-9, D). After 24 hours, the cells in A appear organized into a highly branched array, while those incubated with blocking antibodies remain either as single cells or in small multicellular clusters (B,C and D). The large dark circles in each of the micrographs is an optical artifact. Bar, 100μm.

Figure 34. MCF-10A cells assemble hemidesmosomes on matrigel but this is inhibited by integrin and laminin-5 antibodies. MCF-10A cells were plated onto matrigel in the presence of control IgG (A), or antibodies which block the function of α6 integrin (GoH3, B), α3 integrin (P1B5, C), or laminin-5 (C2-9, D). After 24 hours the cells on matrigel were fixed for electron microscopy. Note that in A there are three hemidesmosome-like structures along the region of cell-matrigel interaction (arrows). One of these (in the box) is shown at higher power in the inset. It possesses an electron dense cytoplasmic plaque and also a sub-basal dense plate. There are no obvious hemidesmosomes in cells in contact with matrigel in B-D (higher power views of these regions are shown in the insets). m, matrigel. Bar in A, 500nm; Bar in inset 60nm.

Table 1. Untreated 804G and MCF10A cells and cells treated with 50 μg/ml of either the laminin-5 function inhibiting antibodies CM6 and P3H9-2 or control IgG were processed for BrdU assay 18 hours after plating. Cells were processed for immunofluorescence and a total of >300 cells counted for each condition in 3 trials. Labeling indices were determined by the percentage of positive BrdU incorporating cells divided by the total number of cells counted under each condition.

Table 2. 804G and MCF10A cells treated with 50 μg/ml of either the laminin-5 function inhibiting antibodies CM6 and P3H9-2 or control IgG for 24 hours were processed for immunofluorescence using BM28 antibody. The percentage of cells at specific stages of the cell cycle are indicated. These were determined according to

Todorov et al. (1995). Note that there is no obvious differences between the CM6 treated 804G cells and their IgG treated counterparts. The same is true for MCF-10A cells.

FIG 1

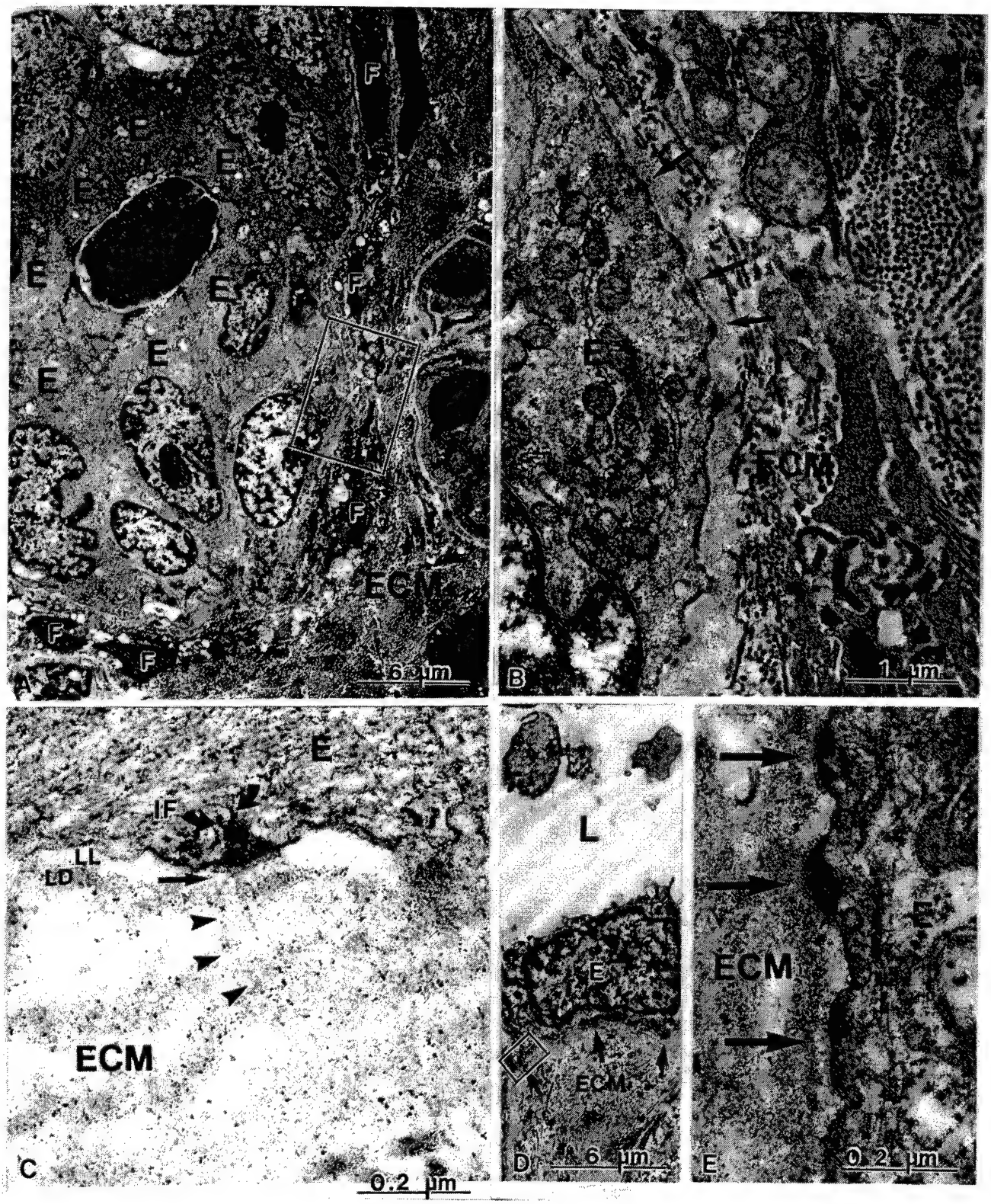

F
E
E
E
E
E
E
E
F
F
F
ECM
F
F
6 µm
A
E
ECM
1 µm
B
E
IF
LL
LD
ECM
C
0.2 µm
L
E
ECM
D
6 µm
E
ECM
E
0.2 µm

FIG 2

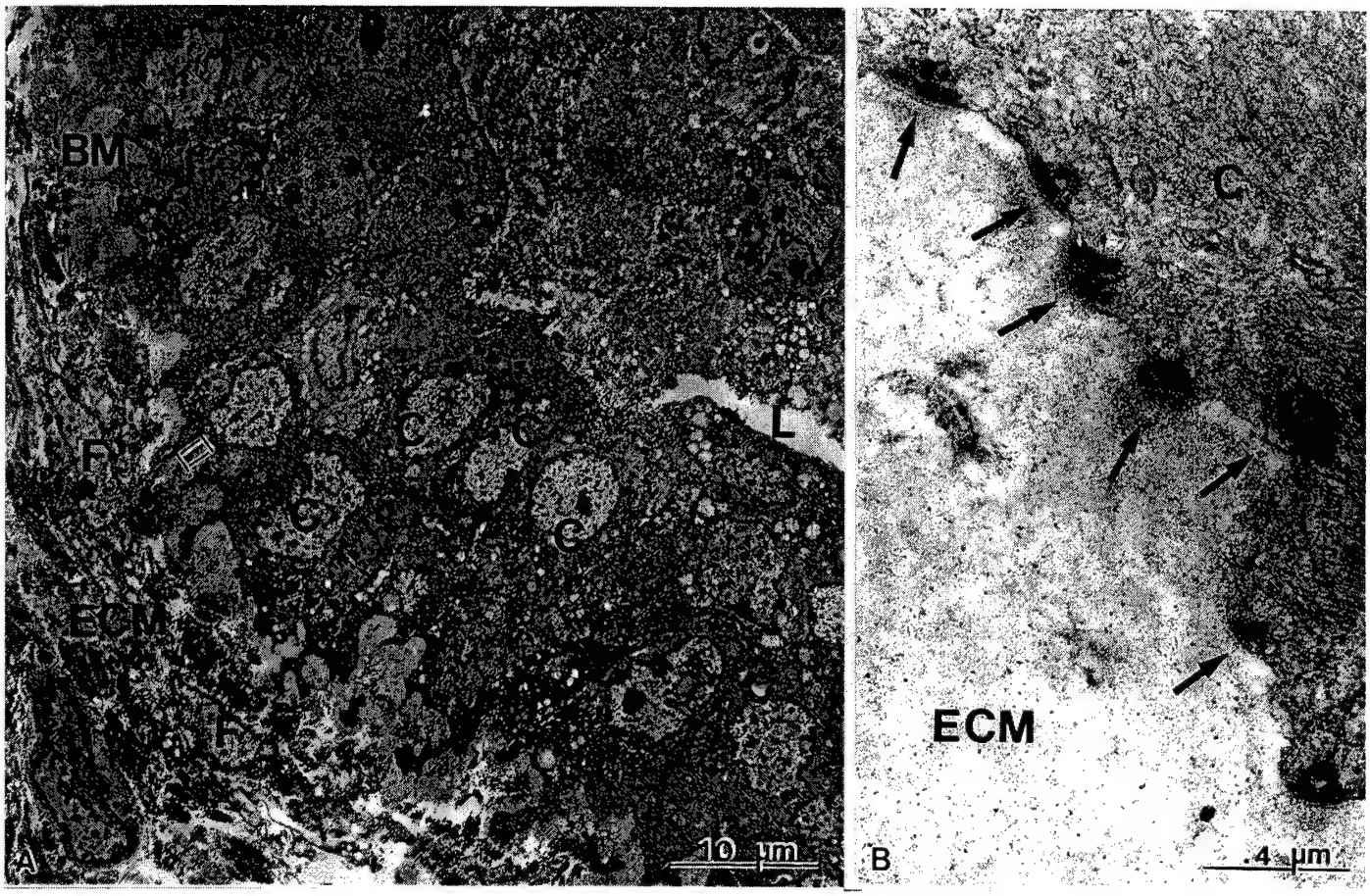


FIG 3

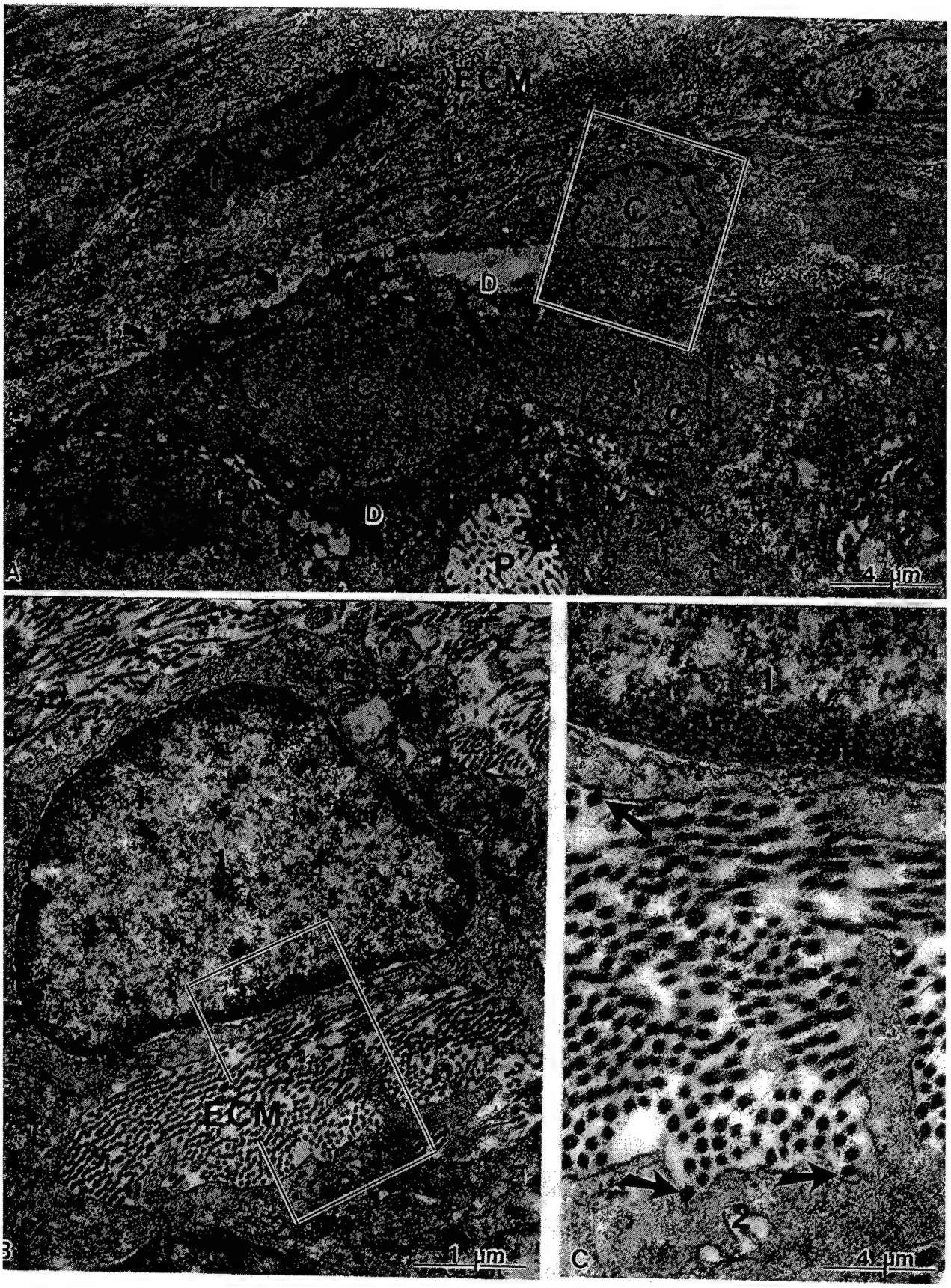


FIG 4

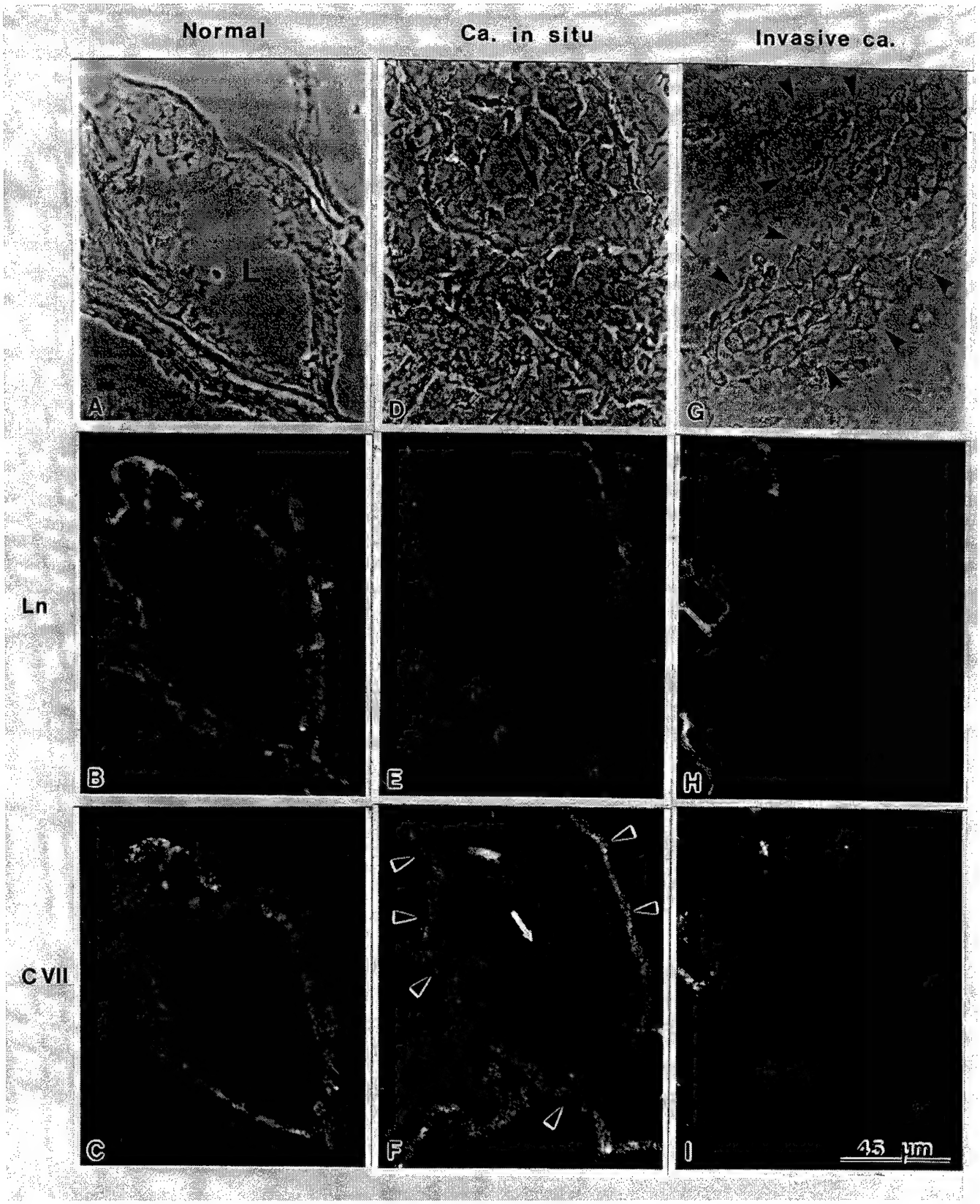


FIG 5

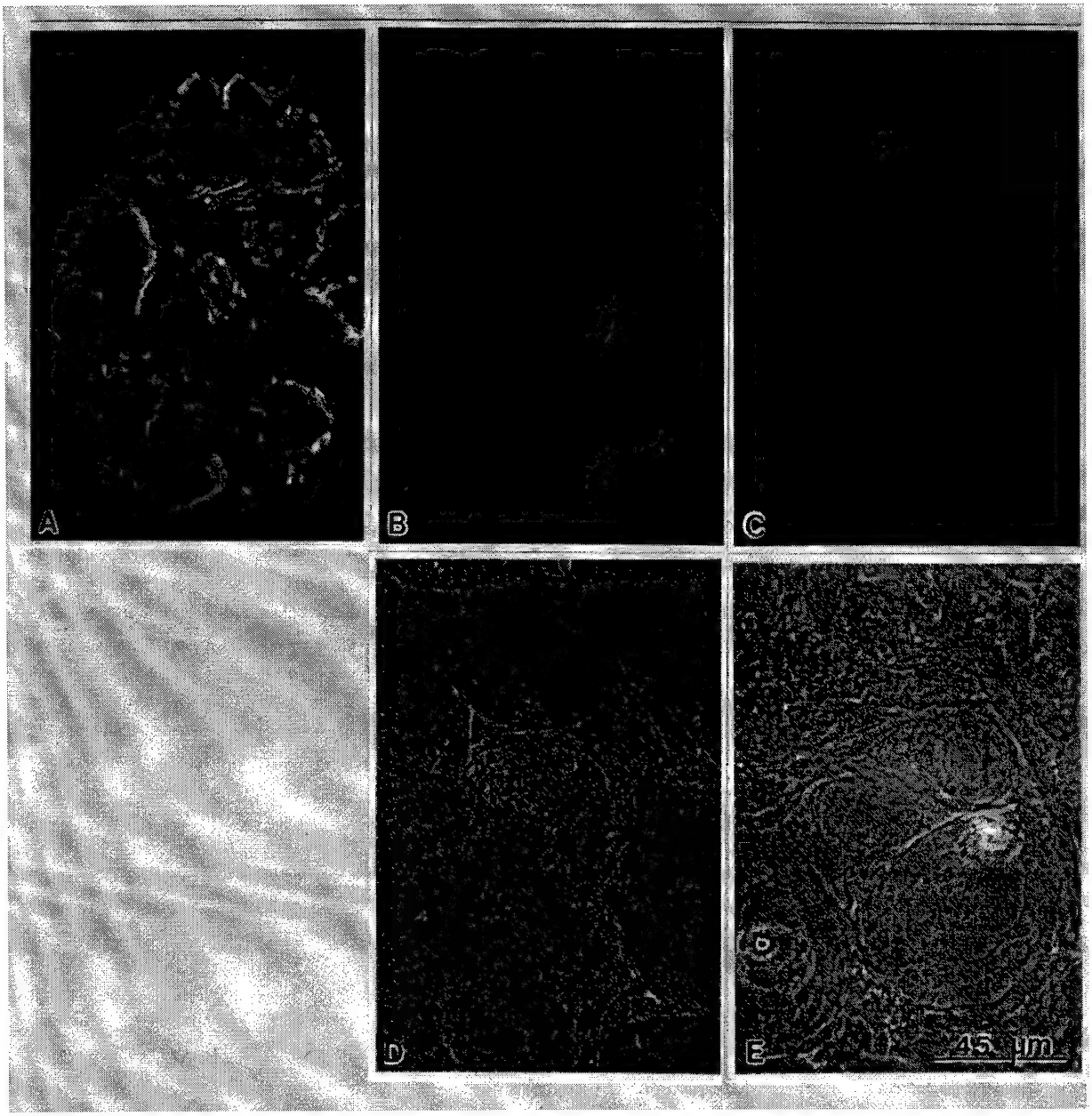


FIG 6

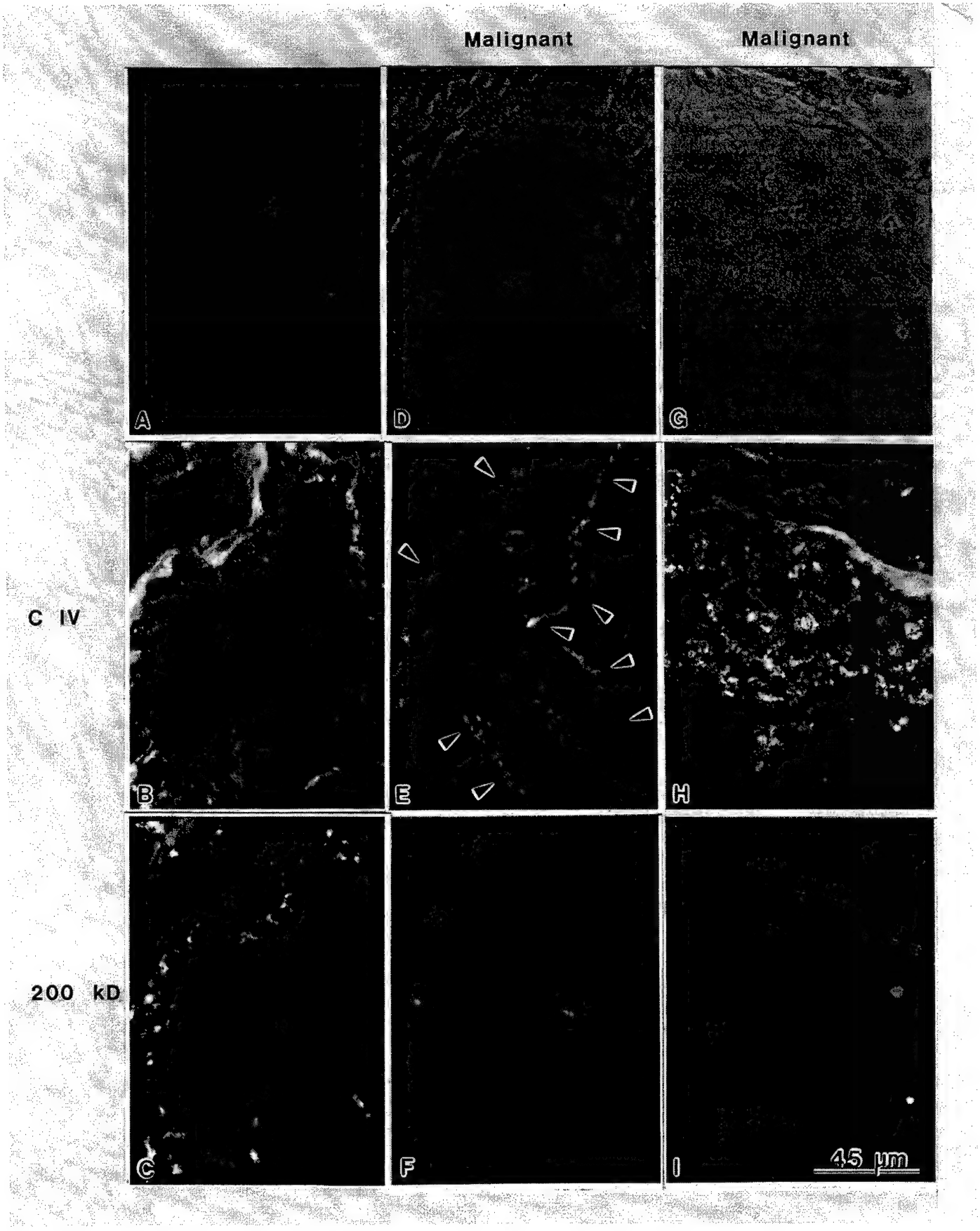


FIG 7

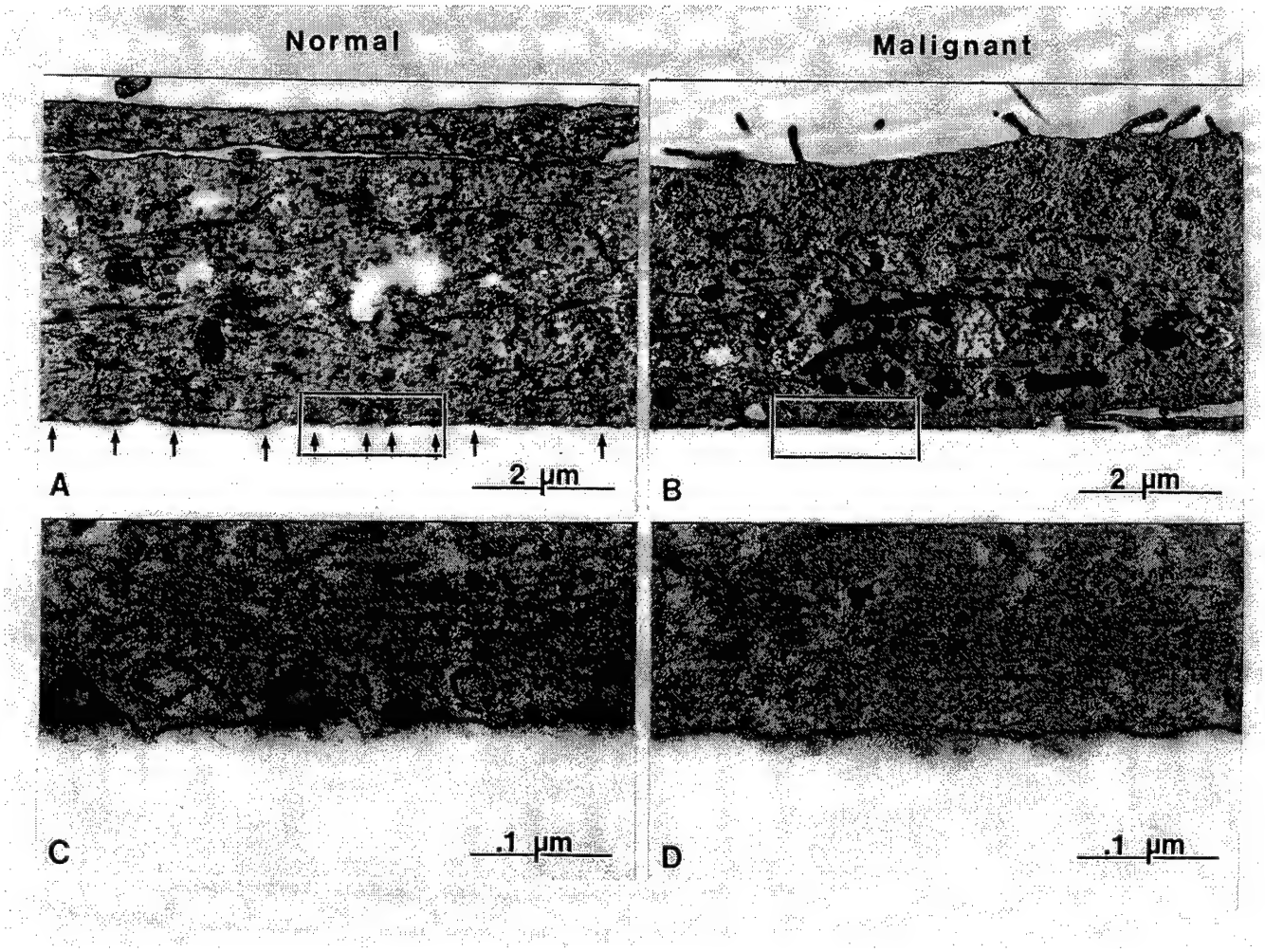


FIG 8

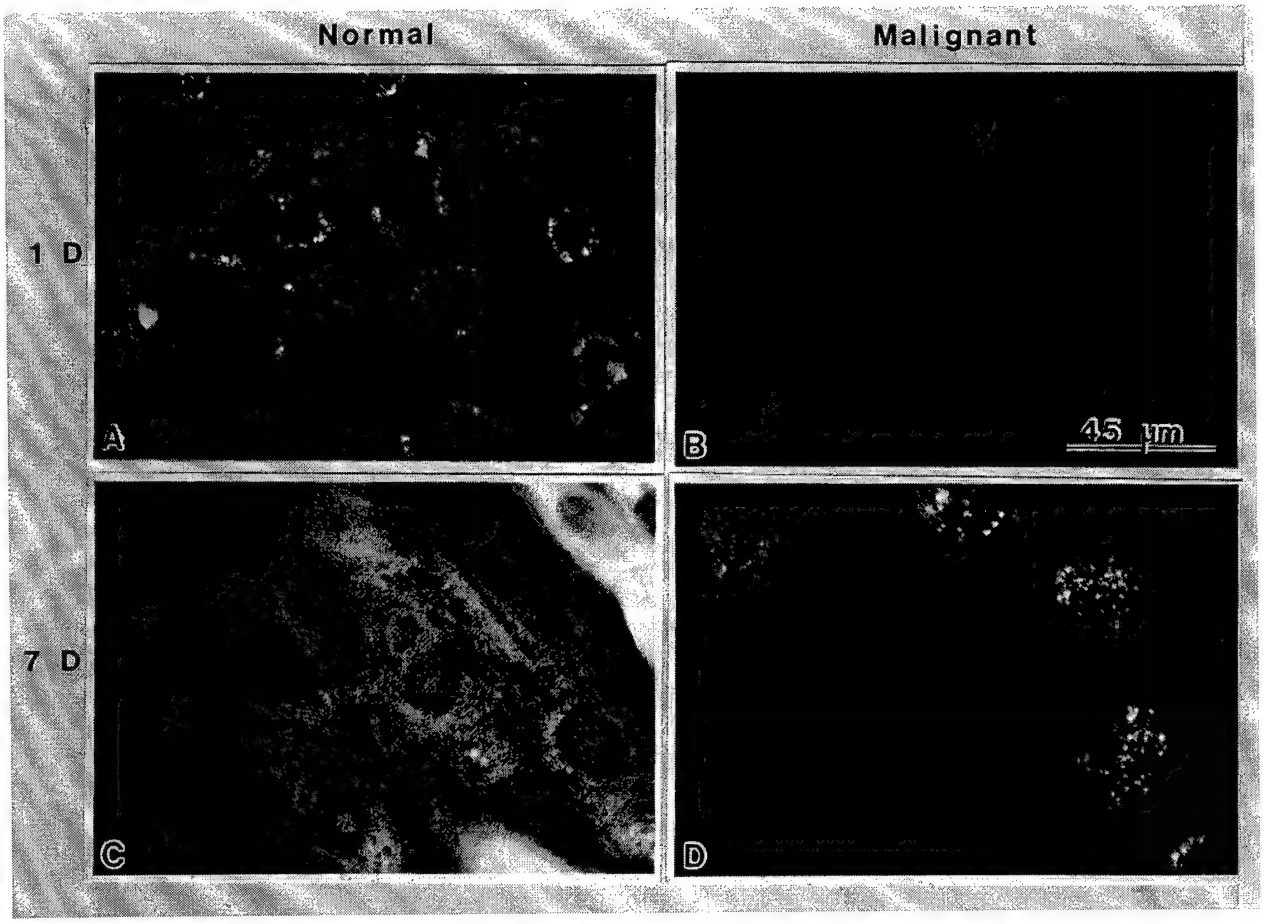


FIG 9

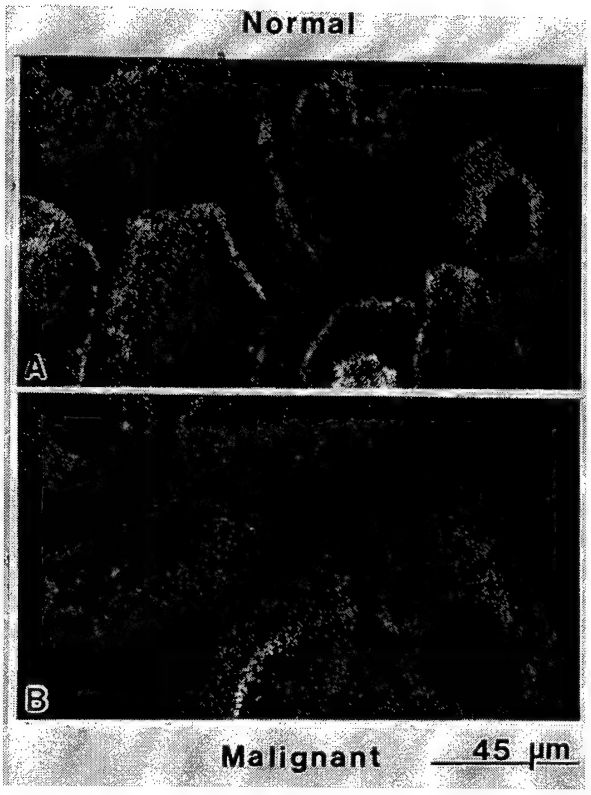


FIG 10

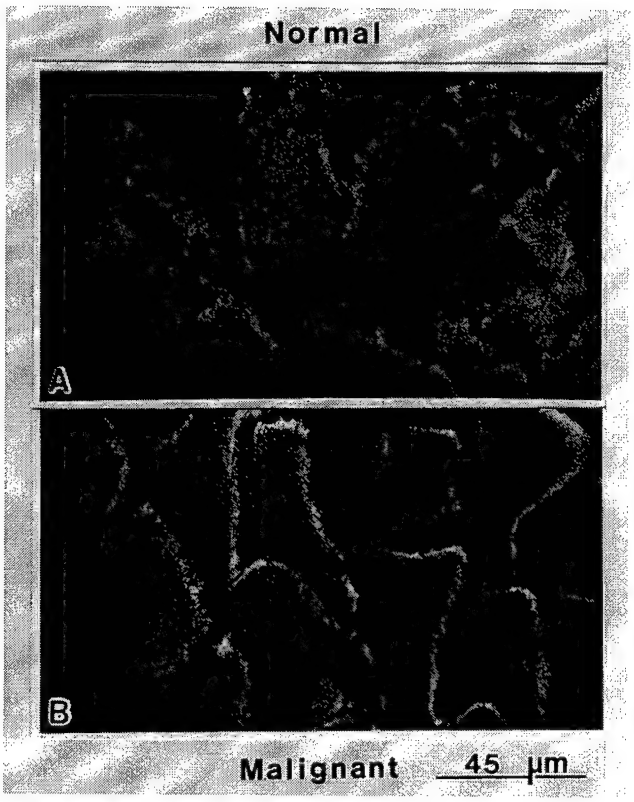


FIG 11

FIG 12

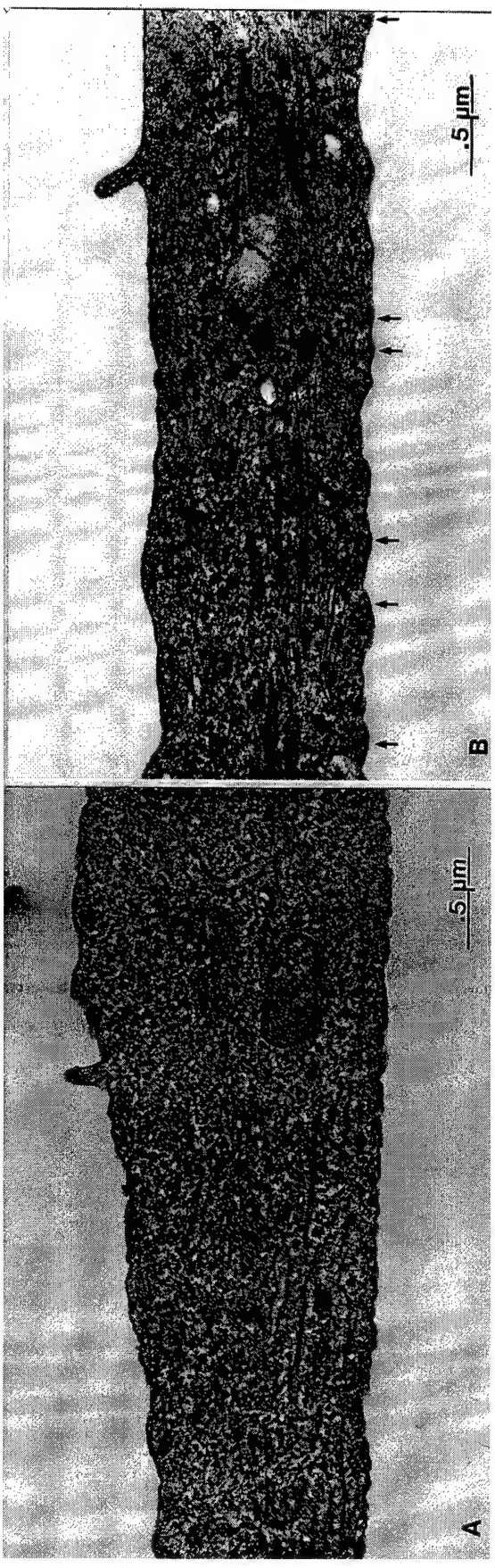


FIG 13

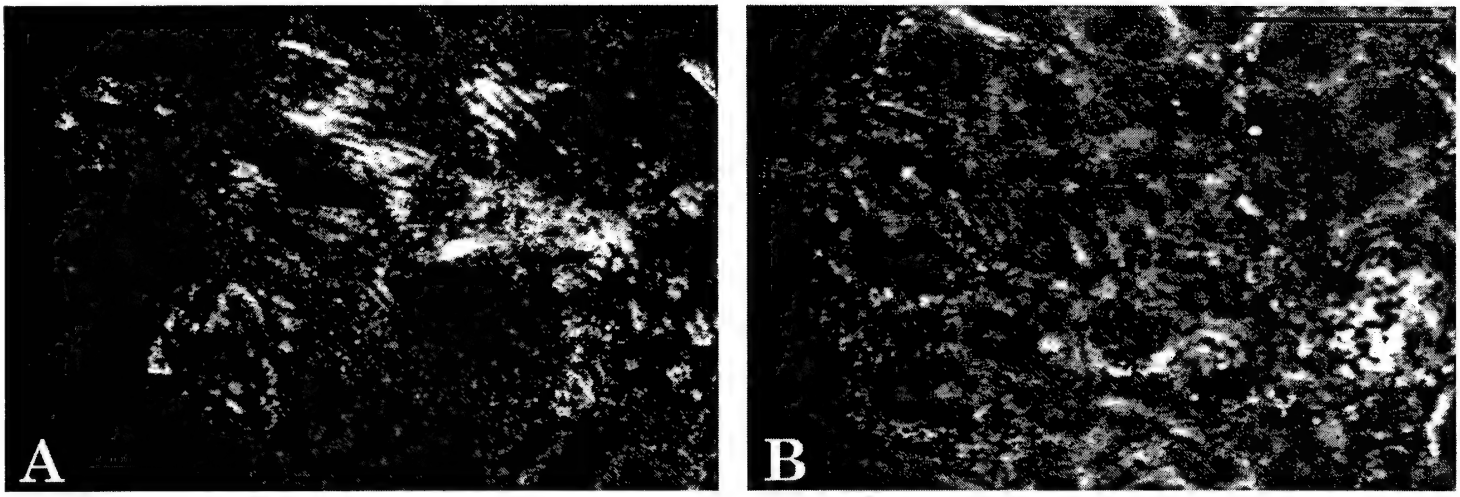


FIG 14

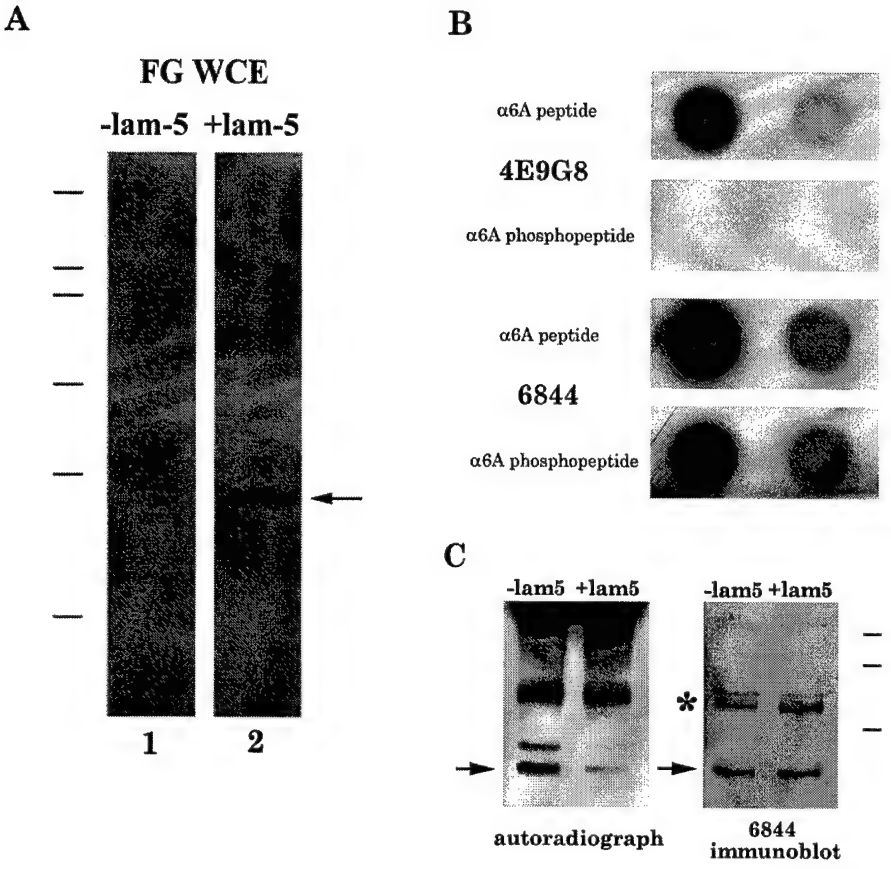

A
FG WCE
-lam-5 +lam-5
1
2
B
α6A peptide
4E9G8
α6A phosphopeptide
α6A peptide
6844
α6A phosphopeptide
C
-lam5 +lam5
-lam5 +lam5
*
autoradiograph
6844
immunoblot

FIG 15

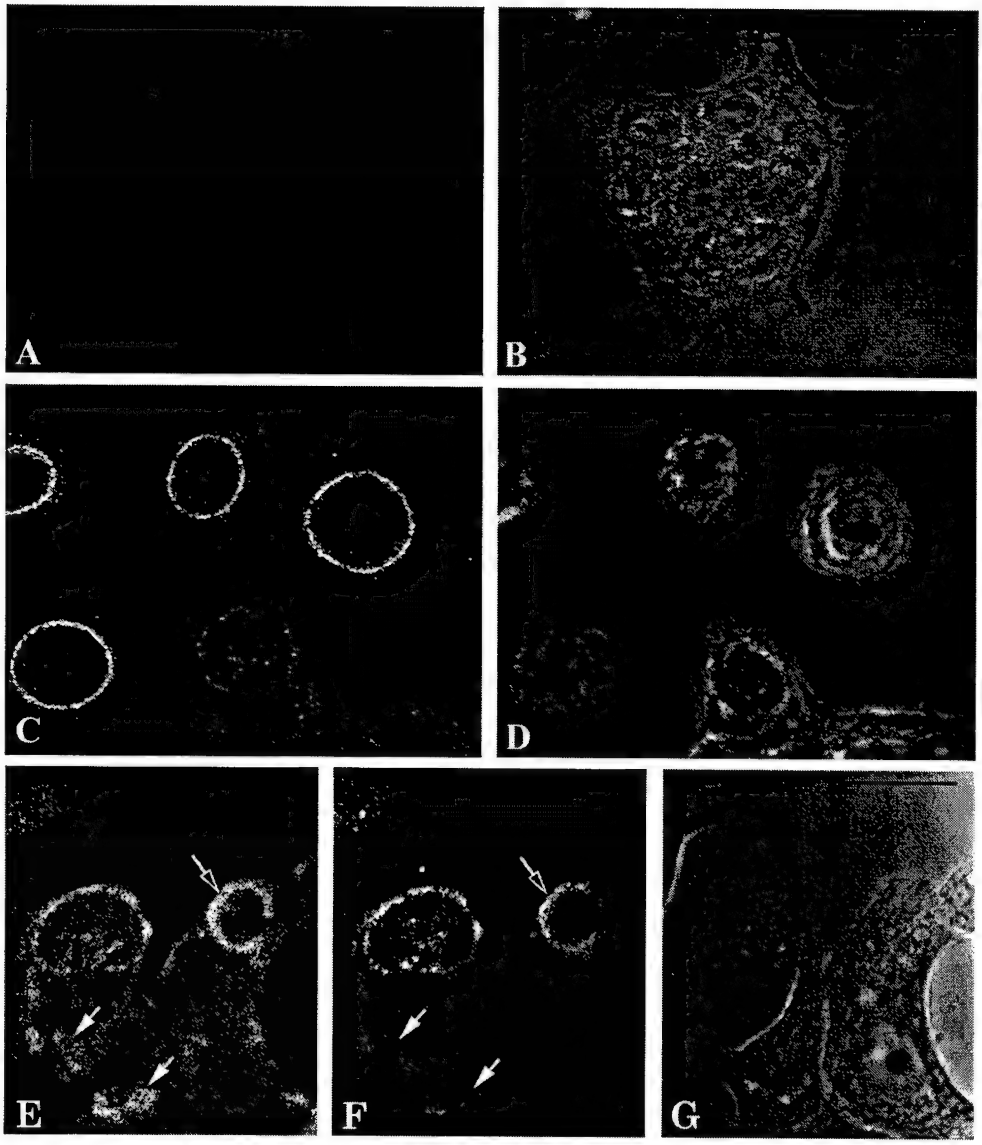
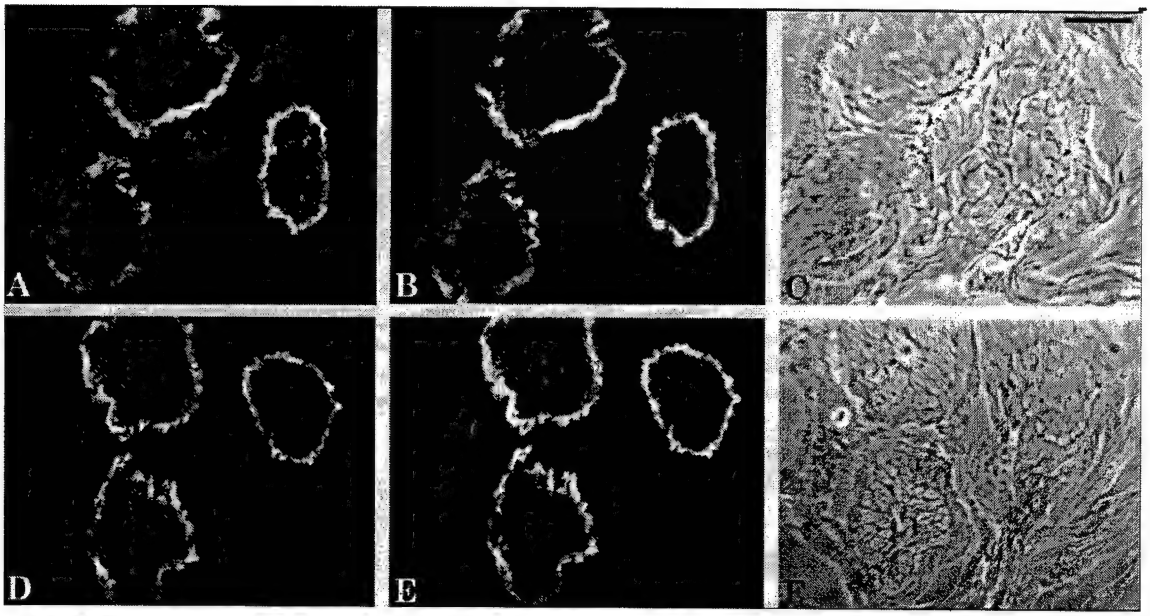


FIG 16



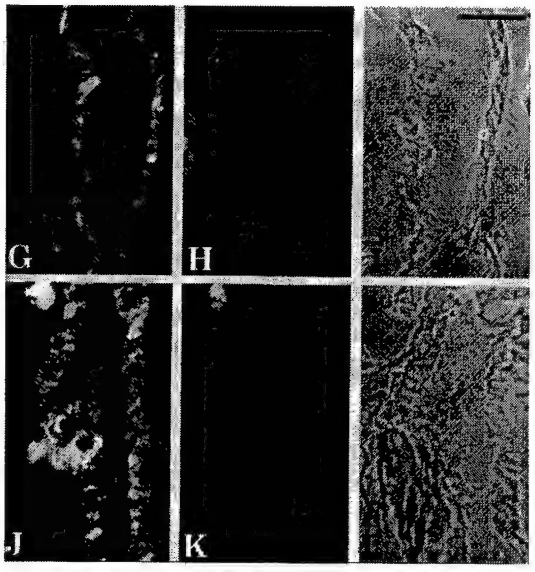
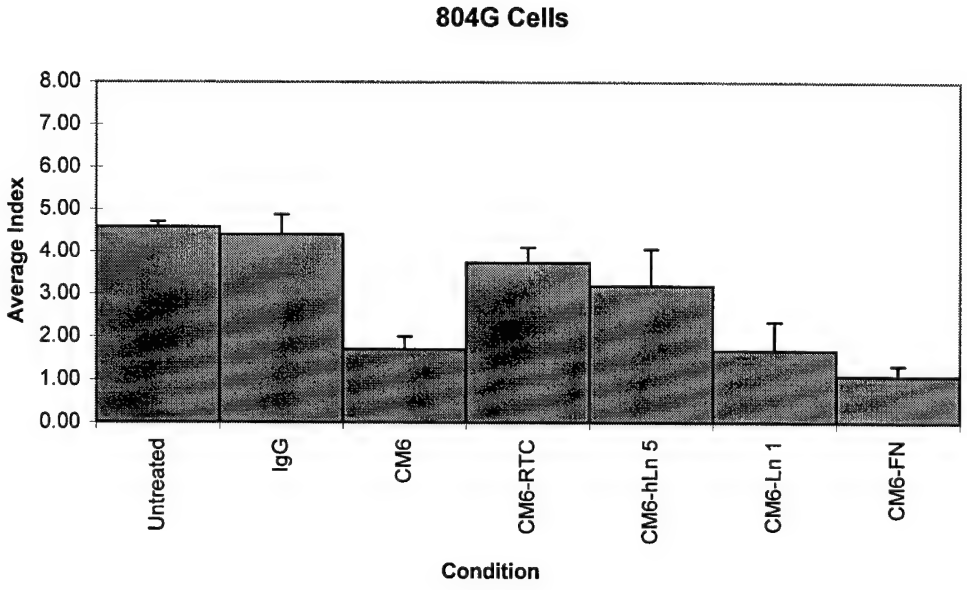


FIG 17


804G Cells
Average Index
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Untreated
IgG
CM6
CM6-RTC
CM6-hLn 5
CM6-Ln 1
CM6-FN
Condition

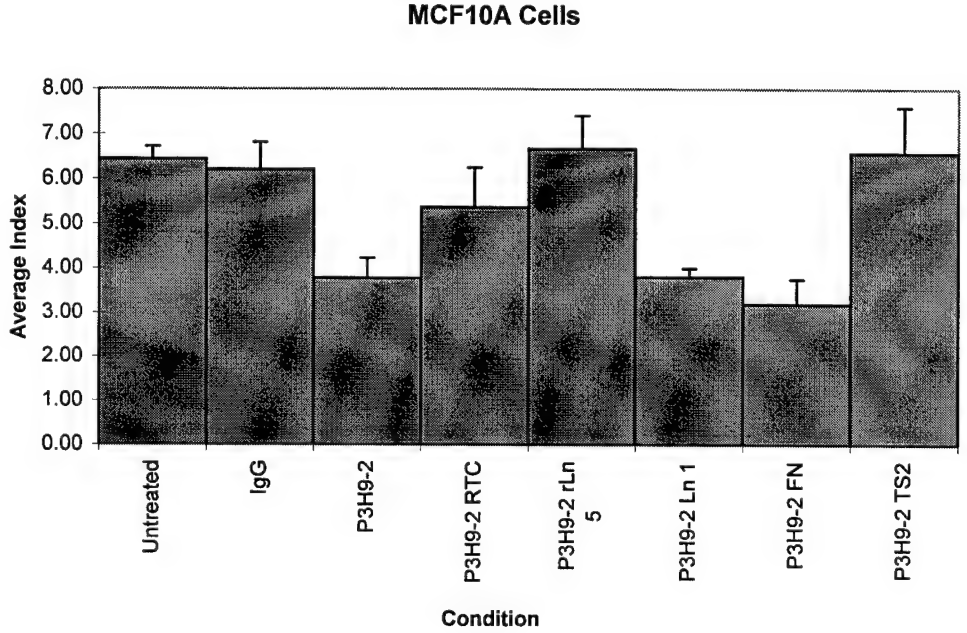

MCF10A Cells
Average Index
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Untreated
IgG
P3H9-2
P3H9-2 RTC
P3H9-2 rLn 5
P3H9-2 Ln 1
P3H9-2 FN
P3H9-2 TS2
Condition

FIG 18

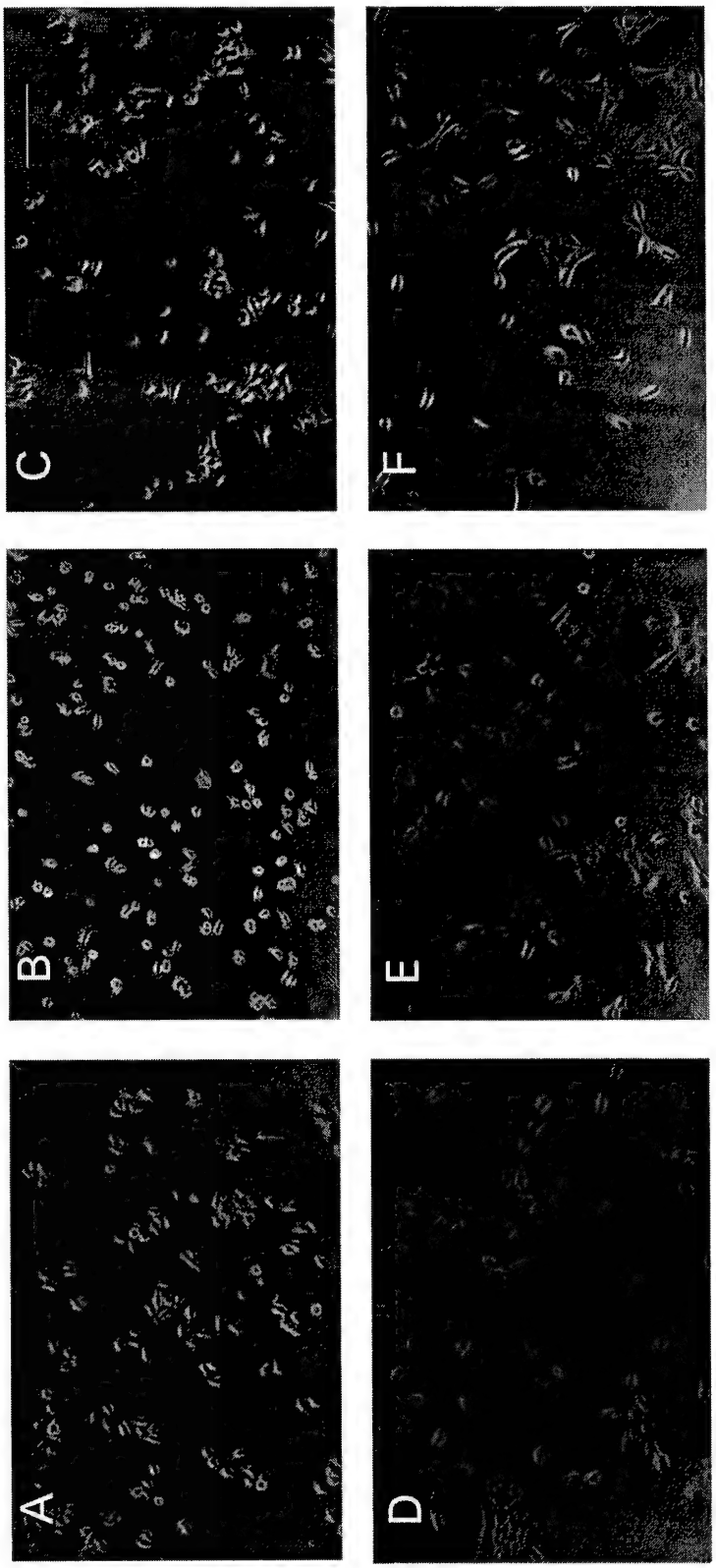


FIG 19

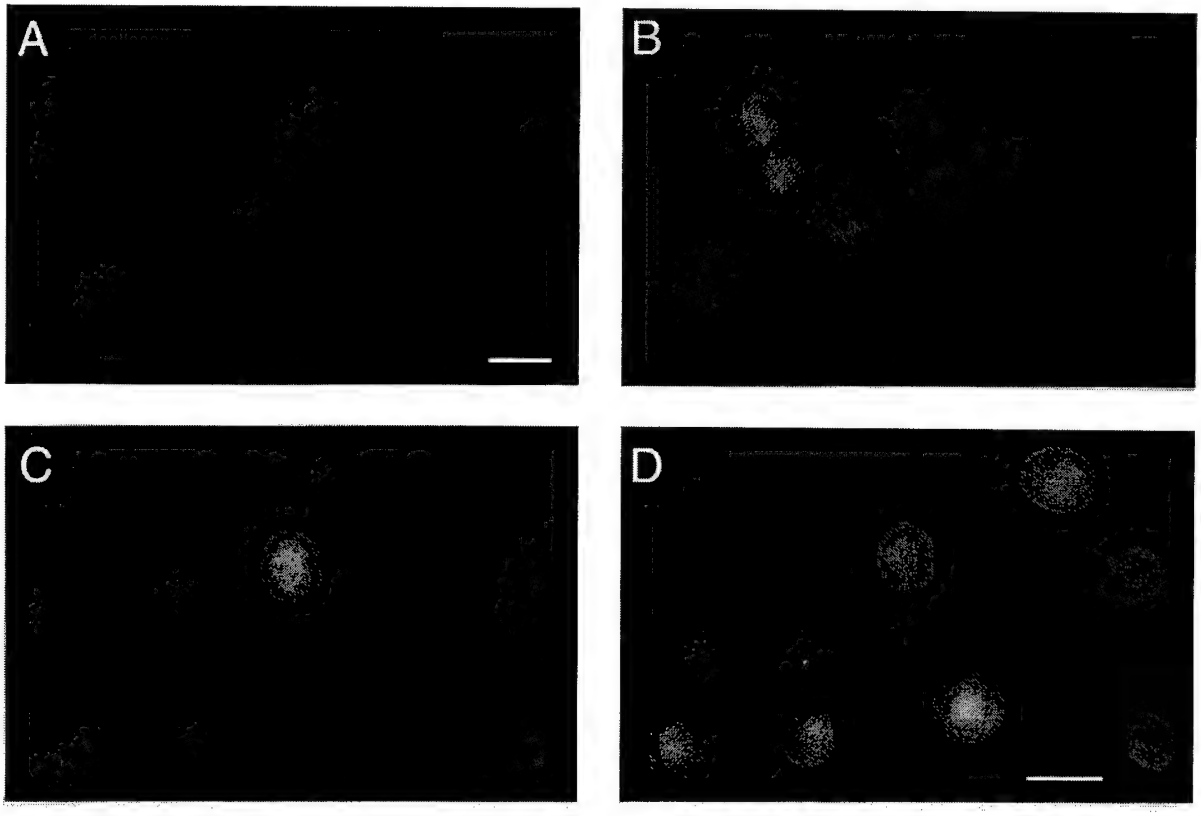


FIG 20

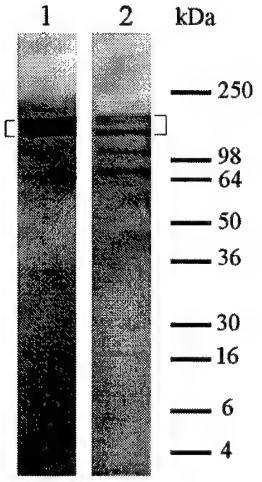


FIG 21

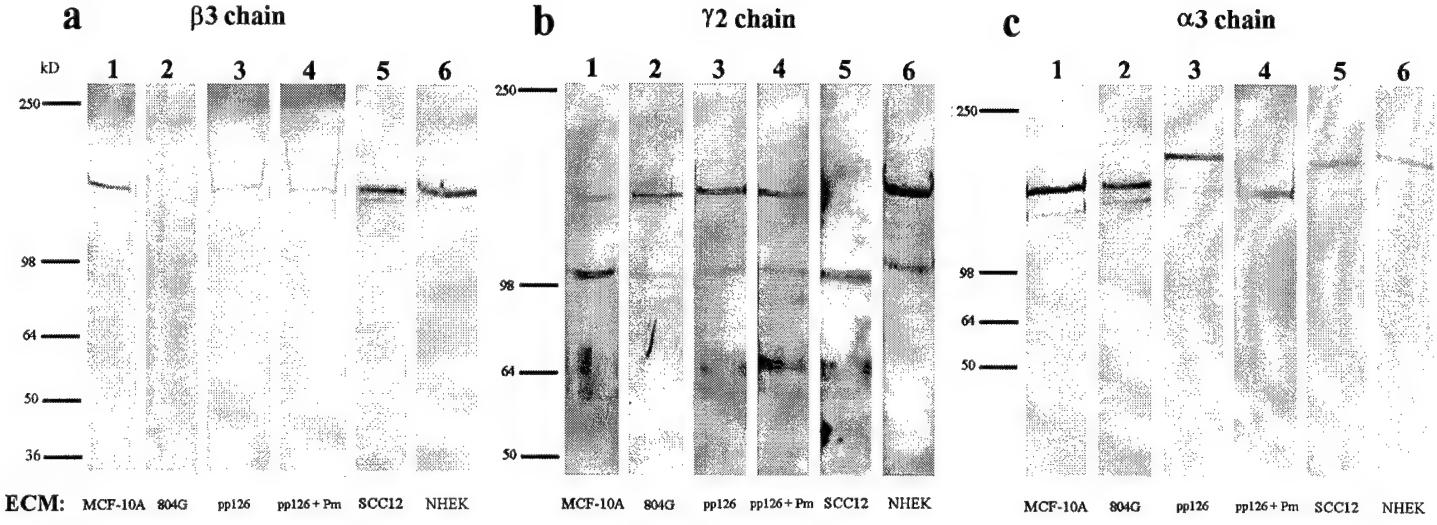

a
β3 chain
b
γ2 chain
c
α3 chain
kD
1
2
3
4
5
6
250
98
64
50
36
ECM:
MCF-10A
804G
pp126
pp126+Pm
SCC12
NHEK

FIG 22

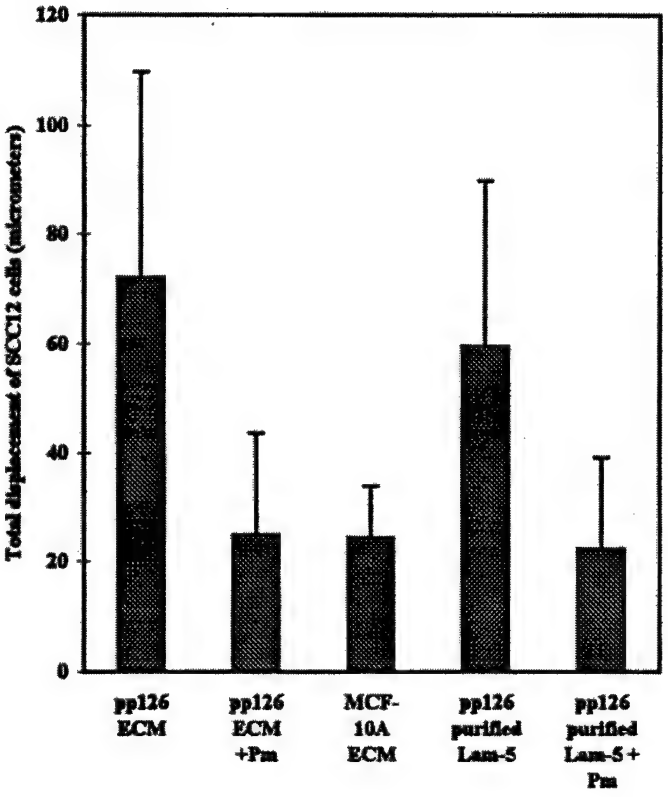

Total displacement of SCC12 cells (micrometers)
120
100
80
60
40
20
0
pp126 ECM
pp126 ECM +Pm
MCF-10A ECM
pp126 purified Lam-5
pp126 purified Lam-5 + Pm

FIG 23

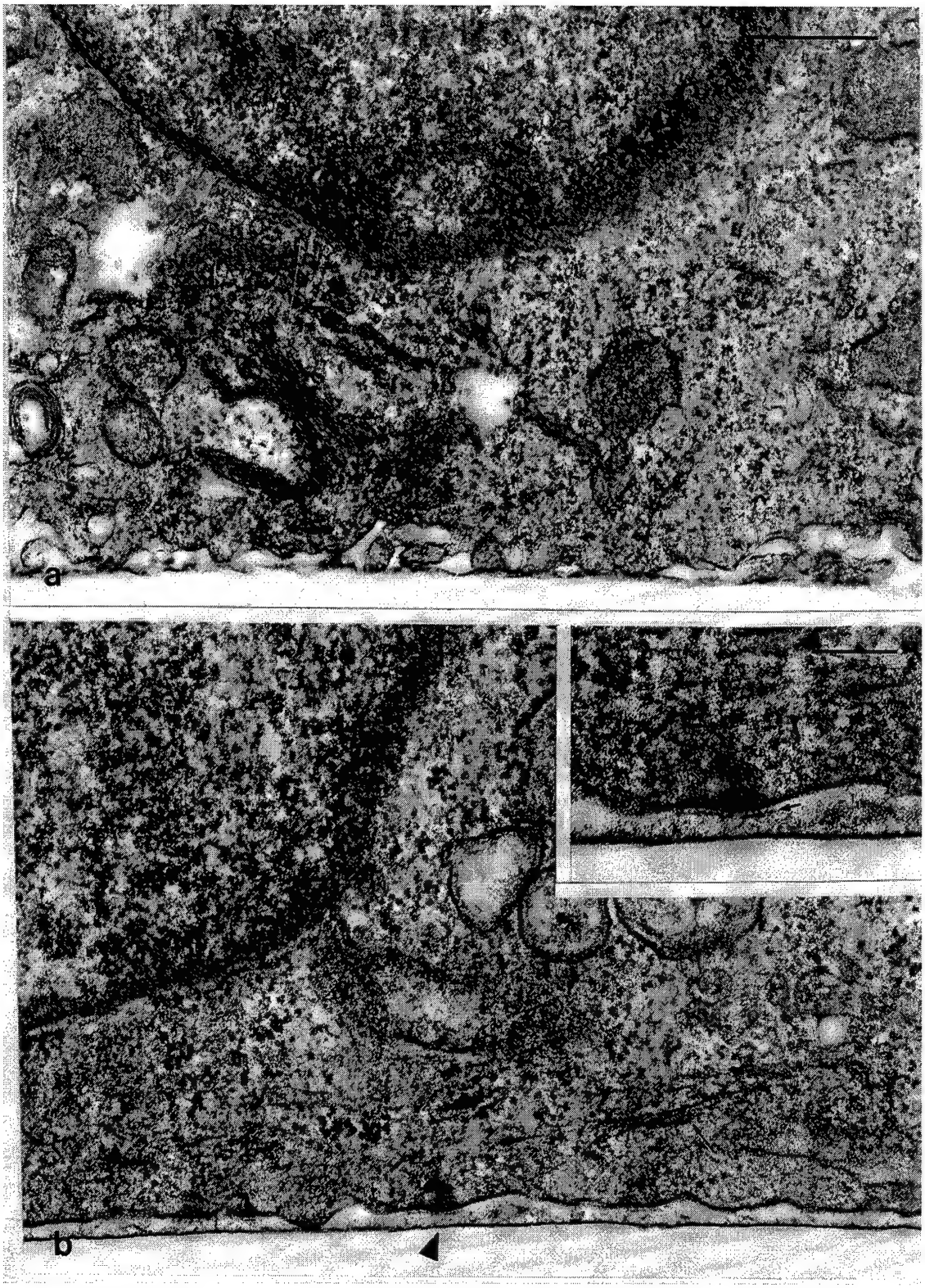


FIG 24

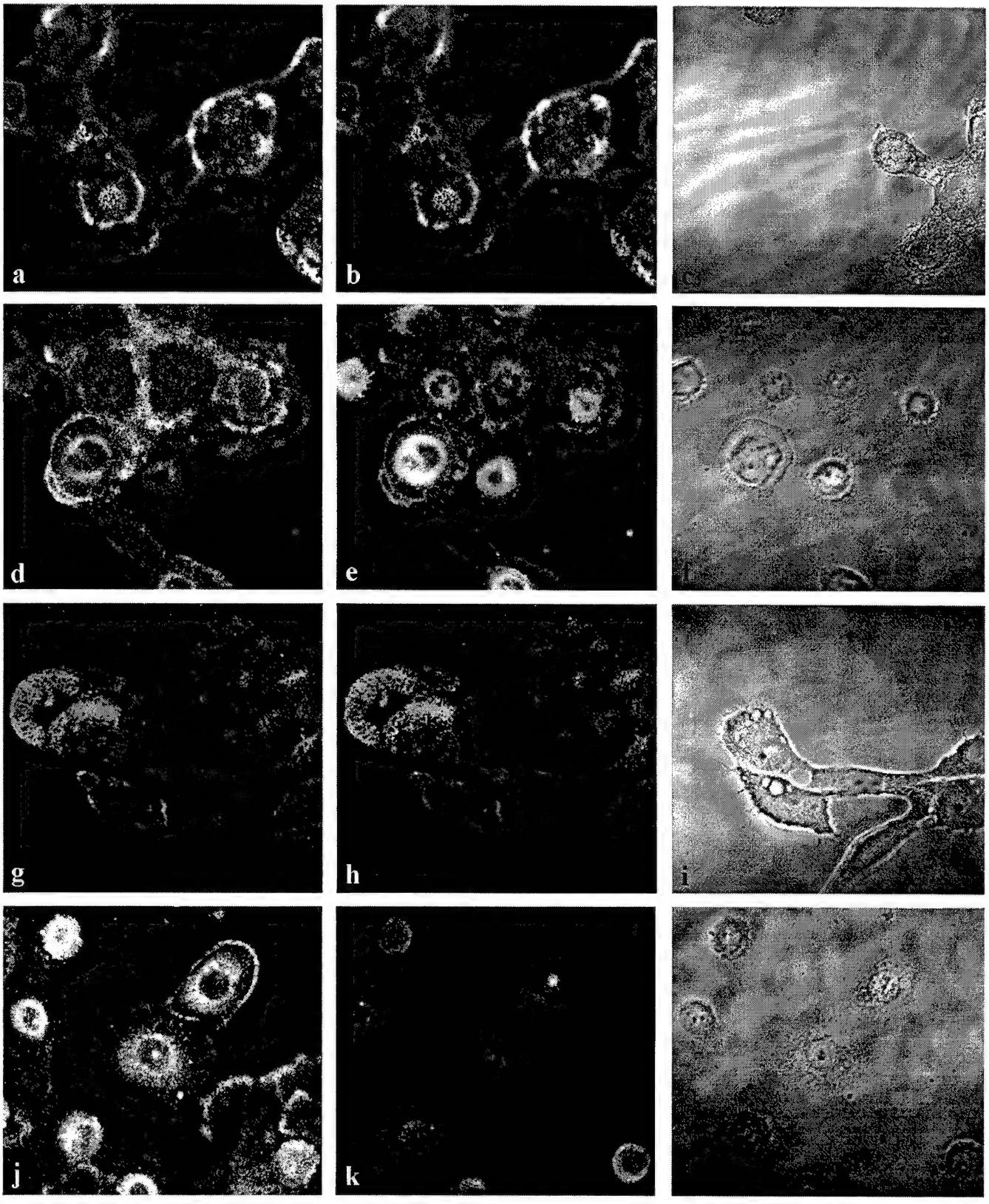


FIG 25

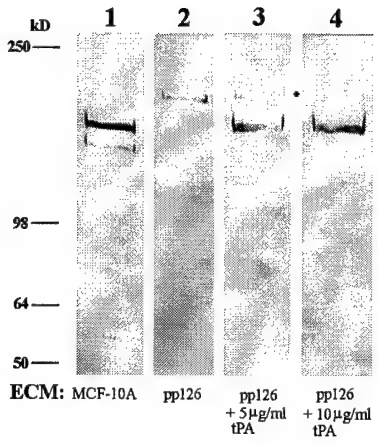

kD
1
2
3
4
250
98
64
50
ECM: MCF-10A
pp126
pp126 + 5µg/ml tPA
pp126 + 10µg/ml tPA

FIG 26

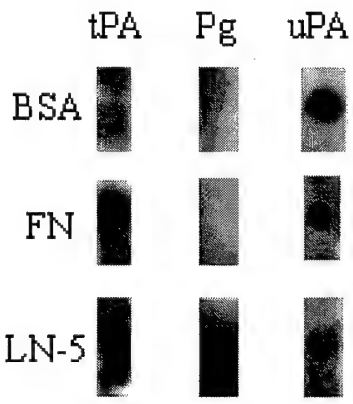


FIG 27

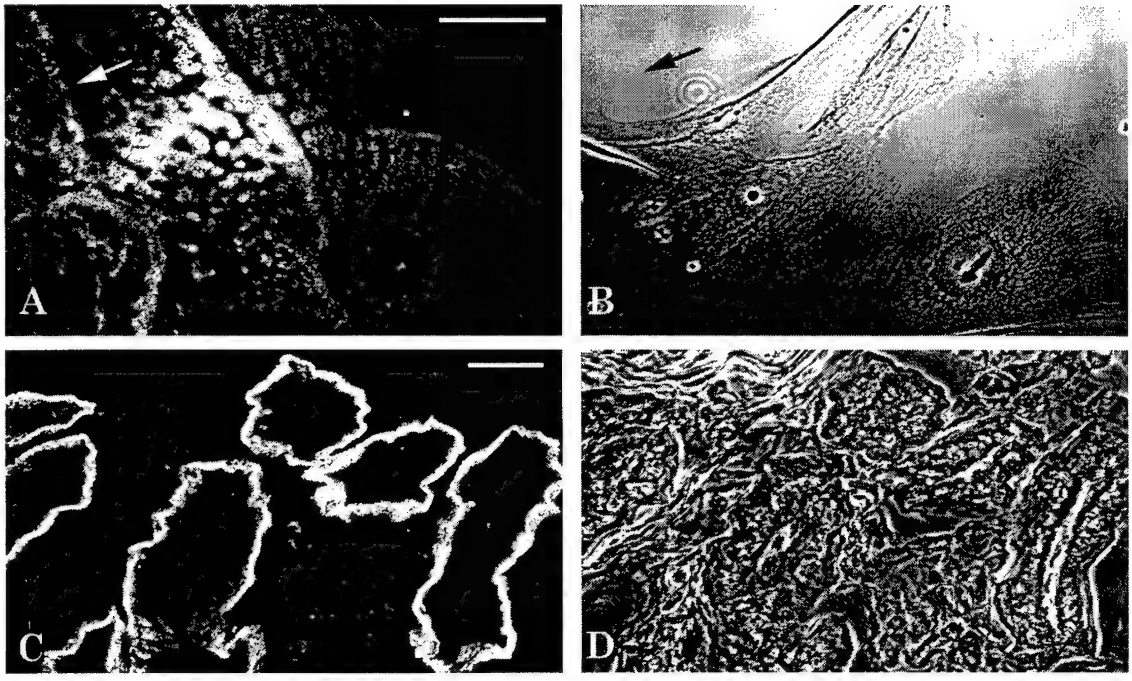


Fig 28

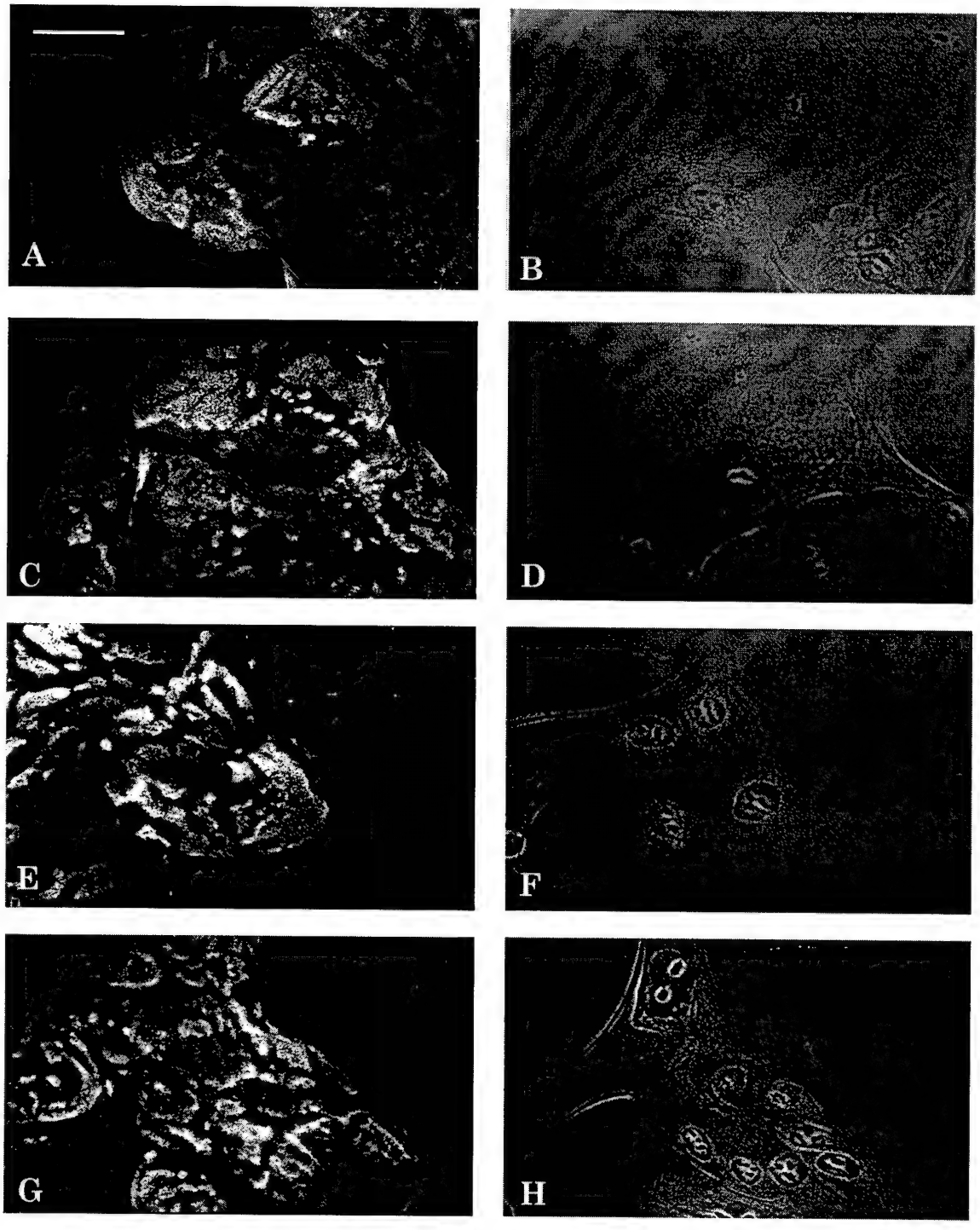


FIG 29

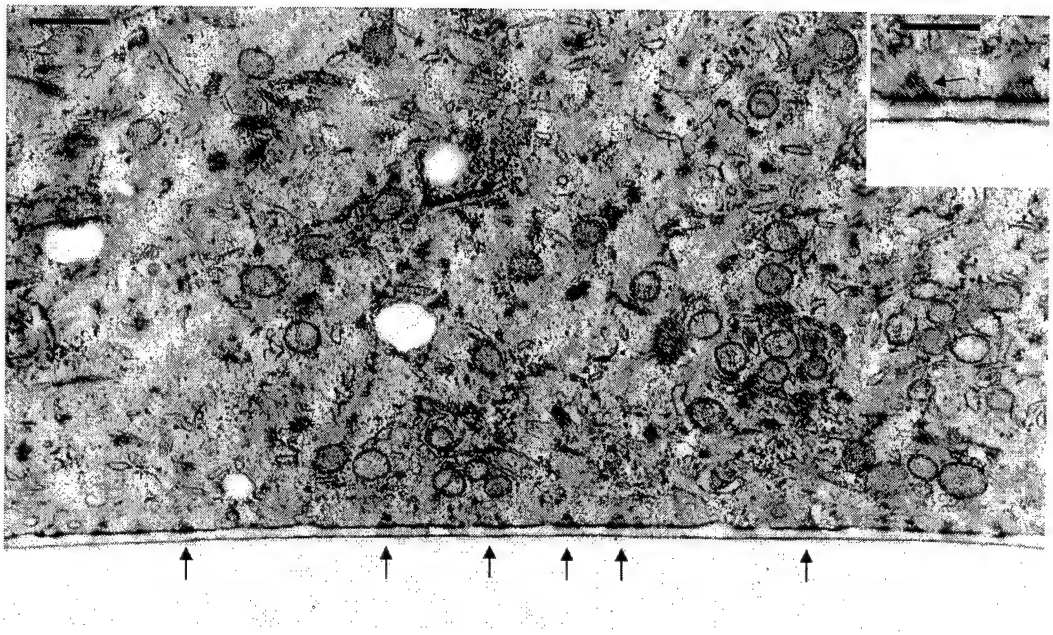


FIG 30

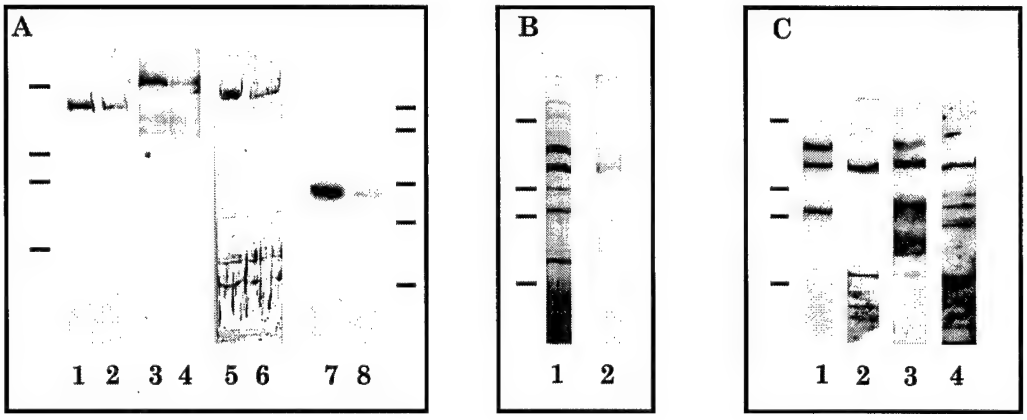


FIG 31

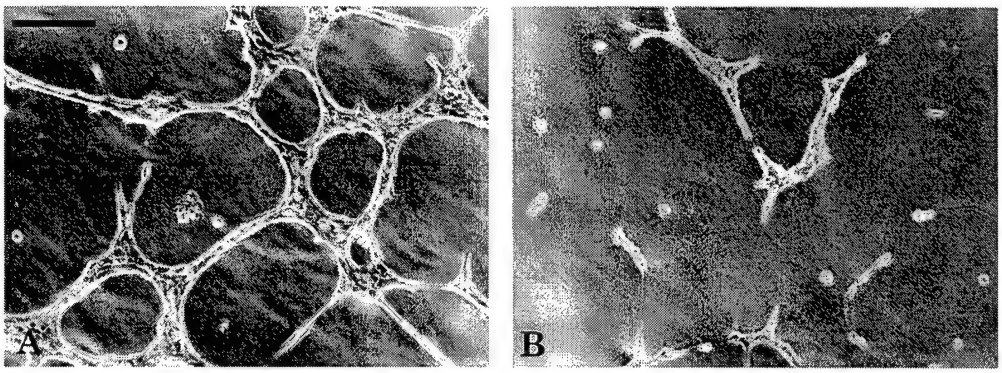


FIG 32

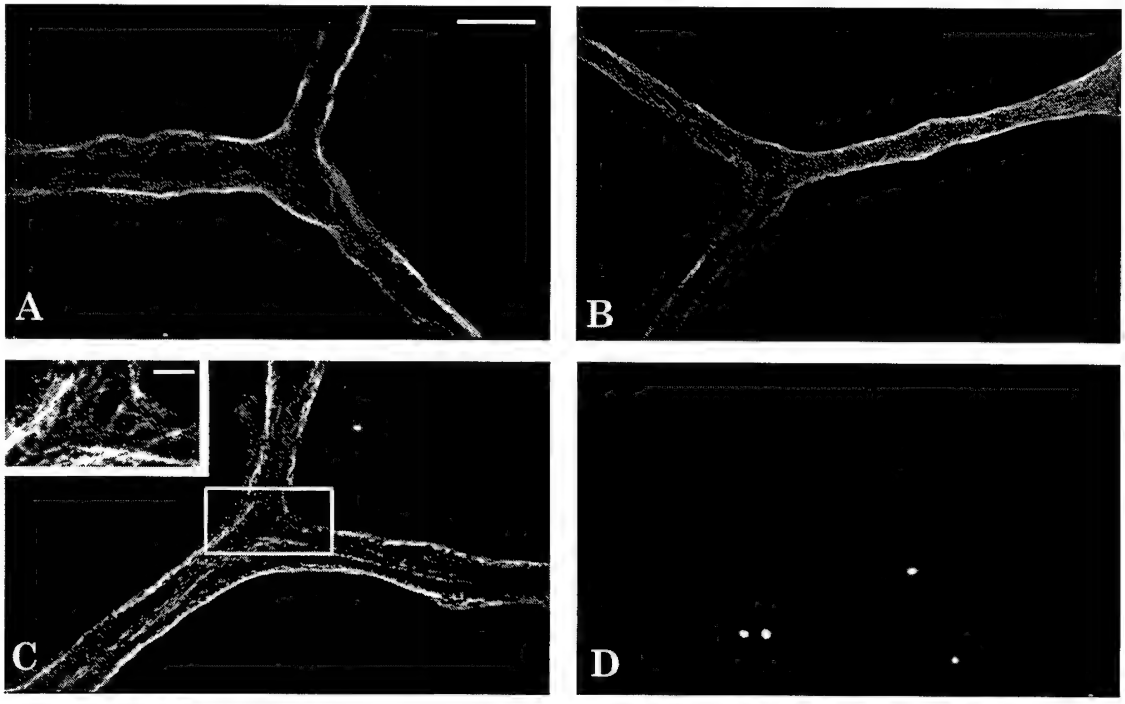


FIG 33

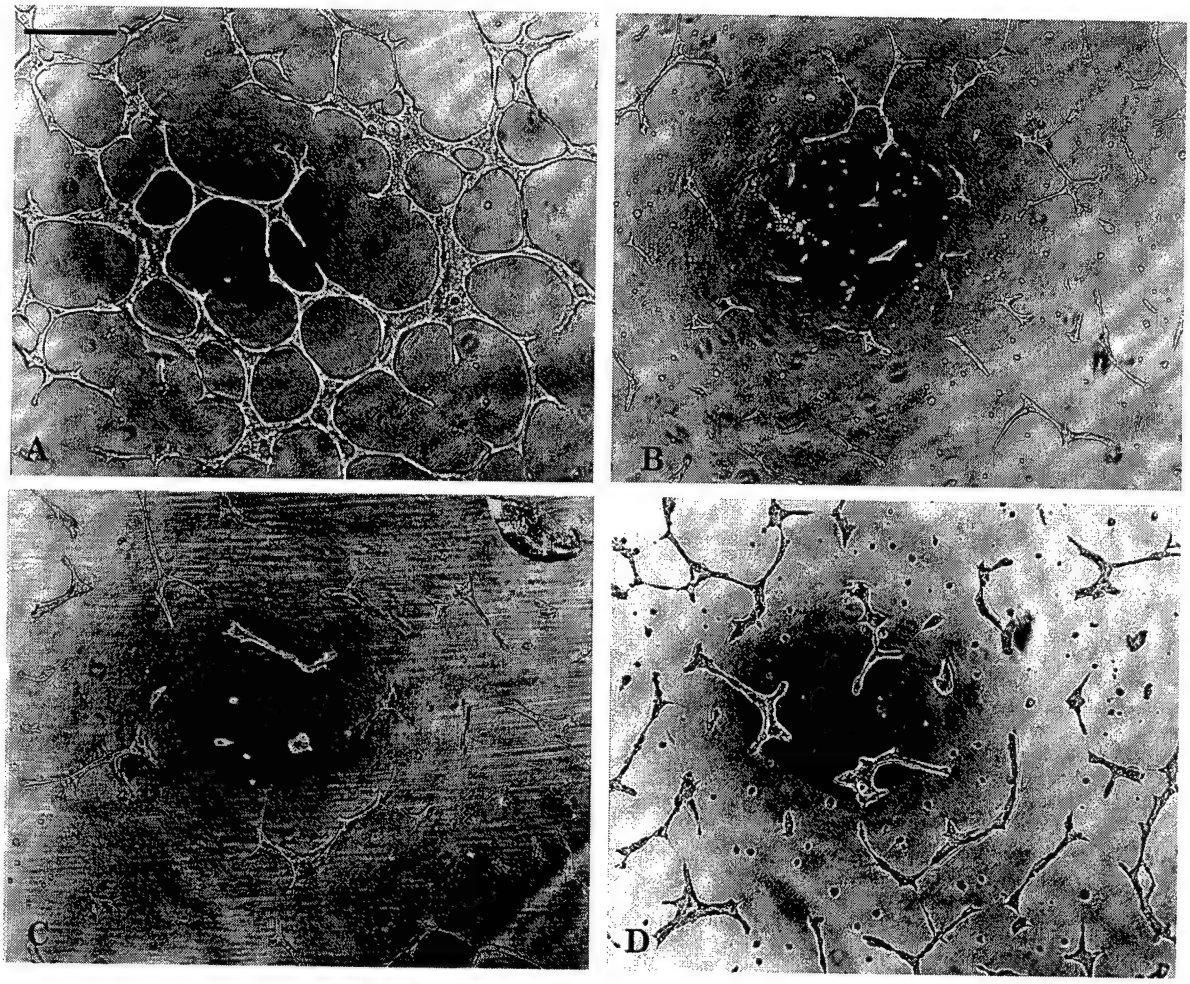


FIG 34

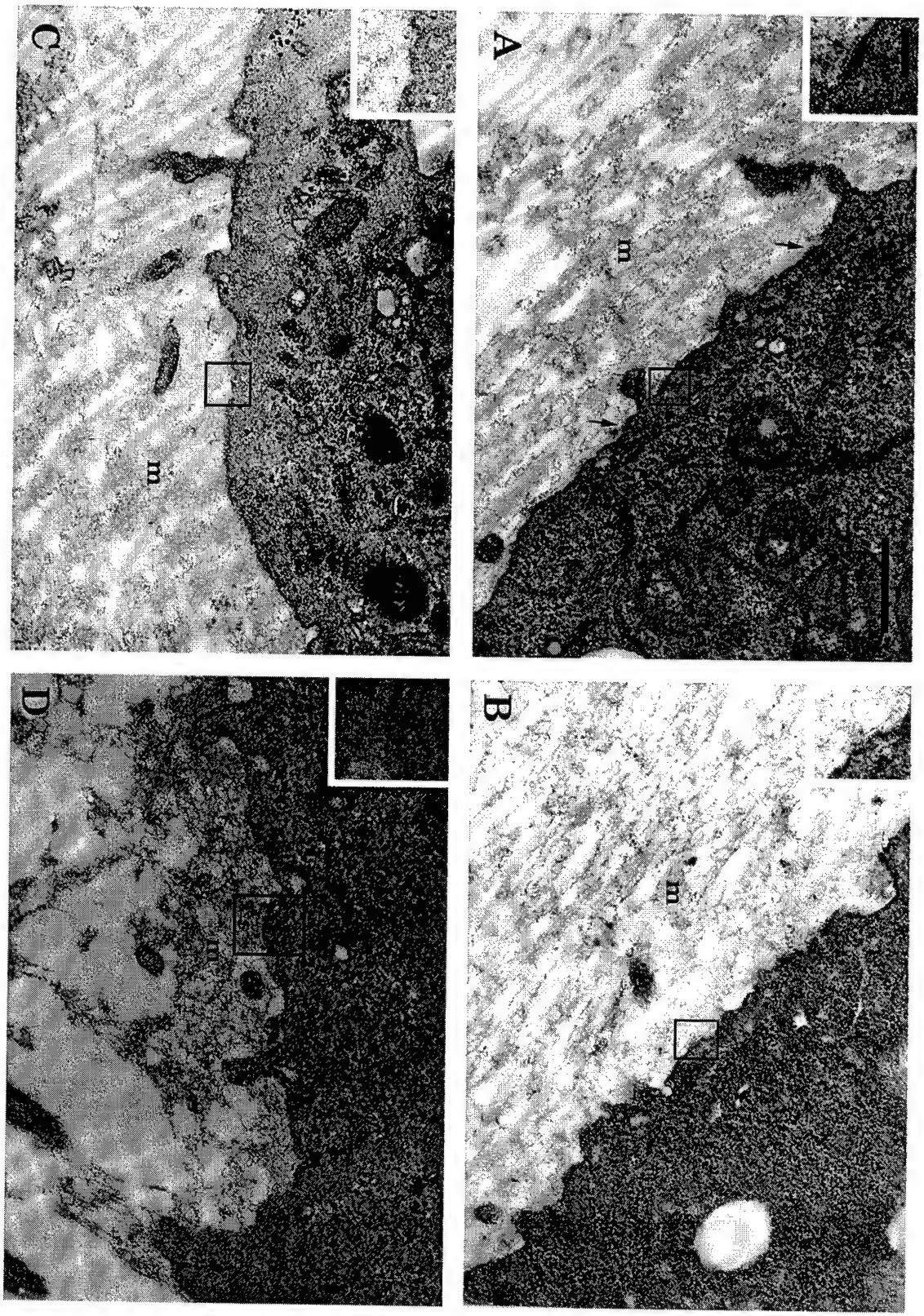


Table I: BrdU Assay

	Cells Counted[1]	Labeling Index (%)
804G Cells		
Untreated	315	48.6
IgG	349	44.4
CM6	357	12.0
MCF10A Cells		
Untreated	619	53.2
IgG	318	57.9
P3H9-2	332	24.4

[1]Total number of cells counted in 3 trials.

Table 2: BM28 Assay

	Cells Counted[1]	G1 (%)	S (%)	G2 (%)
804G Cells				
IgG	371	43.8	33.7	22.4
CM6	210	44.3	38.5	17.2
MCF10A Cells				
IgG	456	43.2	34.0	22.8
P3H9-2	343	40.8	33.5	25.7

[1]Total number of cells counted in 3 trials.

(7) Conclusions

1. Breast epithelial cells utilize hemidesmosomes to attach to the BMZ in normal tissue.
2. Hemidesmosomes are lost in epithelial cells of invasive tumors of the breast.
3. Certain hemidesmosome antibody probes (those that can differentiate between phosphorylated and non- phosphorylated α6 integrin) may be useful in differentiating non-invasive from invasive carcinoma cells.
4. Normal and some tumor cells derived from breast tissue assemble hemidesmosomes in vitro but only after a considerable period in culture.
5. In contrast to normal breast cells, MCF-10A cells readily assemble hemidesmosomes in vitro (within a matter of hours). Like normal breast epithelial cells MCF-10A cells undergo branching morphogenesis in matrigel. Indeed, the MCF-10A cell line is an ideal model for breast morphogenesis studies as well as studies on hemidesmosome assembly.
6. Branching morphogenesis of MCF-10A cells is inhibited by antibodies to laminin-5 and its receptors indicating a role for hemidesmosome components in normal breast tissue development.
7. MCF-10A cells process the α chain of laminin-5 in a very specific way involving the plasminogen/tPA system. This processing plays a crtical role in the ability of laminin-5 to nucleate hemidesmosome assembly.
8. Laminin-5 is involved in epithelial cell proliferation via signalling events transduced by its integrin receptors.

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(9) **Appendix**

We include the following papers as part of our progress report:

Bergstraesser, L.M, Srinivasan, G., Jones, J.C.R., Stahl, S. and Weitzman, S.A. (1995). Expression of hemidesmosomes and component proteins is lost by invasive breast cancer cells. Am. J. Path., 147:1823-1839.

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Baker, S.E., Hopkinson, S.B., Fitchmun, M., Andreason, G.L., Frasier, F., Plopper, G., Quaranta, V., and Jones, J.C.R. (1996) Laminin-5 and hemidesmosomes: role of the α3 chain subunit in hemidesmosome stability and assembly. J. Cell Sci., 109:2509-2520.

Baker, S.E., DiPasquale, A., Stock, E.L., Plopper, G., Quaranta, V., Fitchmun, M. and Jones J.C.R. (1996) Morphogenetic effects and utility in organ culture of a soluble laminin variant, laminin-5. Exp. Cell Res., 228:262-270.

Stahl, S., Weitzman, S. and Jones. J.C.R. (1997) The role of laminin-5 and its receptors in mammary epithelial cell branching morphogenesis. J. Cell Sci., 110:55-63.

Baker, S.E., Skalli, O., Goldman, R.D. and Jones, J.C.R. (1997) Laminin-5 and modulation of keratin cytoskeleton arrangement in FG pancreatic carcinoma cells: involvement of IFAP300 and evidence that laminin-5/cell interactions correlate with a dephosphorylation of α6A integrin. Cell Motil. Cyto., 37:271-286.

Jones, J.C.R.., Gonzales, M., Hopkinson, S.B. and Goldfinger, L. Structure and assembly of hemidesmosomes. Bioessays, in press.

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Appendix Materials

Jonathan Jones

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Laminin-5 and hemidesmosomes: role of the α3 chain subunit in hemidesmosome stability and assembly

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SUMMARY

Hemidesmosomes are complex macromolecular structures which integrate elements of the extracellular matrix and the cytoskeleton of epithelial cells. To characterize cell-matrix interactions in the hemidesmosome, we have made use of 804G cells which possess the unusual ability to assemble hemidesmosomes in vitro. During the course of our studies, we have raised a set of monoclonal antibodies against rat laminin-5, the major structural element comprising 804G matrix. One of these, termed CM6, recognizes the 150 kDa α chain of rat laminin-5 and binds the globular (G) domain of intact laminin-5 molecules as determined by rotary shadowing. CM6 antibodies perturb formed hemidesmosomes in 804G cells. In particular, within 1 hour of incubation of 804G cells with CM6 antibodies, colocalization of laminin-5 and α6β4 integrin is lost and by 2 hours, staining generated by hemidesmosomal antibodies appears primarily cytoplasmic in the perinuclear zone. Ultrastructurally, CM6 antibodies first appear to induce detachment of hemidesmosomes from the underlying matrix. Next, portions of the basal cell surface invaginate to form vesicles whose cytoplasmic-facing surface is coated with hemidesmosomes still associated with keratin intermediate filaments. Anchoring filaments extend into the inside compartment of the vesicles. We have also studied the impact of CM6 antibodies on a model system in which the matrix of 804G cells induces de novo assembly of hemidesmosomes in human keratinocytes. This process involves the plasma membrane reorganization of the hemidesmosome associated integrin α6β4 as well as a redistribution of other hemidesmosome components such as the 230 kDa bullous pemphigoid antigen. Pretreatment of 804G matrix with CM6 antibodies blocks such plasma membrane reorganization of hemidesmosome components and inhibits hemidesmosome formation. Our studies indicate a crucial role for the G domain of the α chain of laminin-5 in both nucleation of hemidesmosome assembly as well as maintenance of hemidesmosome structural integrity.

Key words: Matrix, Integrin, Adhesion, Basement membrane

INTRODUCTION

The basement membrane, composed of a complex mix of several extracellular matrix components, including collagens, laminins and proteoglycans, acts as a physical barrier which separates epithelial cells from connective tissue. In addition, it plays an important role in regulating morphogenesis of epithelial tissues (Strueli et al., 1995). Although basement membranes in a variety of tissue types appear morphologically similar in the electron microscope, it is becoming increasingly clear that they show differences in their biochemical composition. For example, the laminins are a family of cruciform shaped molecules which display tissue specific patterns of expression (Tryggvason, 1993). Laminin-2 (merosin) is a component of the basement membranes of placenta, striated muscle and peripheral nerve (Ehrig et al., 1990). Laminin-5 is expressed in basement membranes of stratified squamous epithelial tissues, transitional epithelium, in the lung and in various epithelial glands (Kallunki et al., 1992; J. C. R. Jones, unpublished observations). Indeed, laminins provide considerable structural diversity to basement membranes and one assumes that this has a major impact on the way a cell interacts with matrix as well as basement membrane function.

Laminin-5 is spatially associated in several epithelial tissue types, including the epidermis, cornea, oral mucosa and mammary gland, with structures called hemidesmosomes (Rousselle et al., 1991; Jones et al., 1994). Hemidesmosomes have been studied extensively at the electron microscope level, but it is only recently that their molecular composition has been investigated (Jones et al., 1994). Moreover, we still have few clues to the function of the protein components of the hemidesmosome. For example, the 230 kDa bullous pemphigoid antigen (BP230) appears to play a role in anchorage of intermediate filaments to the cytoplasmic plaque of the hemidesmosome while the 180 kDa bullous pemphigoid antigen (BP180) is involved in adhesion of epithelial cells to the basement membrane (Liu et al., 1993; Jones et al., 1994; Guo et al., 1995; Hopkinson et al., 1995). In addition to BP180,

the integrin heterodimer α6β4 mediates hemidesmosome interactions with extracellular matrix (De Luca et al., 1990; Stepp et al., 1990; Jones et al., 1991; Sonnenberg et al., 1991; Kurpakus et al., 1991). Intriguingly, Mainiero et al. (1995) have recently speculated that α6β4 integrin may be involved in transduction of signals from matrix to an epithelial cell and vice versa based on their finding that Shc and Grb2 are associated with β4 integrin in A431 cells.

Previous studies from our laboratory and others have provided evidence that the α6β4 integrin heterodimer plays a critical role in assembly of hemidesmosomes (Jones et al., 1991; Kurpakus et al., 1991; Spinardi et al., 1995). Moreover, the ability of α6β4 integrin to nucleate hemidesmosome assembly requires participation of extracellular matrix components as described by Langhofer et al. (1993). In the latter study, keratinocytes were induced to form hemidesmosomes by maintaining them on matrix secreted by a rat bladder cell line termed 804G. This induction correlates with a spatial reorganization of α6β4 integrin as well as both BP antigens. At the biochemical level, the major component of 804G matrix is laminin-5, prompting Langhofer et al. (1993) to speculate that laminin-5 is the ligand for the α6β4 integrin and acts in concert with α6β4 to 'trigger' hemidesmosome assembly. This conclusion, however, is circumstantial since no direct evidence has yet been presented that directly links laminin-5 and induction of hemidesmosome formation.

In order to test the hypothesis that laminin-5 is involved in hemidesmosome formation, we have generated a set of monoclonal antibodies against 804G cell matrix components. Reactive antibodies were screened for their ability to functionally inhibit the properties of 804G cell matrix. During the course of our studies we have identified one antibody called CM6 which, as we detail here, disrupts assembled hemidesmosomes in 804G cells. Furthermore, CM6 antibodies block matrix-induced de novo hemidesmosome assembly in SCC12 cells. We discuss our study in light of recent evidence that the pathogenesis of certain blistering skin diseases results from mutations in laminin-5 expression. We speculate that such mutations inhibit hemidesmosome assembly thereby weakening epithelial cell-matrix interactions.

MATERIALS AND METHODS

Cell culture and preparation of a laminin-5 rich matrix

SCC12 cells and 804G cells were cultured as detailed previously (Langhofer et al., 1993; Riddelle et al., 1991). 804G cell matrix, rich in laminin-5, was prepared as described previously (Gospodarowicz, 1984; Langhofer et al., 1993). Matrix preparations were washed extensively in PBS prior to addition of SCC12 cells in KGM (Cascade Biologicals, Portland, OR). In some instances 804G matrix was incubated in PBS containing 50 μg/ml of matrix monoclonal antibodies (see below) for 45 minutes at 37°C. Antibody treated matrix was washed extensively in PBS before addition of SCC12 cells. In some of our experiments, 804G cells were maintained for 48 hours on glass coverslips. Matrix monoclonal antibodies were then added to the medium of the 804G cells to a concentration of 50 μg/ml and the cells were incubated in the antibody containing medium for a further 2 hours.

Antibodies

5E is a human monoclonal antibody against BP230 and was a kind gift from Dr Takashi Hashimoto (Keio University, Tokyo, Japan). The hybridoma line producing 5E antibodies was made by fusing Epstein-Barr virus transformed peripheral B cells of a bullous pemphigoid patient with cells of the human lymphoid B cell line AC33 (Hashimoto et al., 1993). Rabbit affinity purified antibodies against the α6A integrin subunit were described by Tamura et al. (1990). GoH3, a rat monoclonal antibody that recognizes the α6 integrin subunit, was purchased from Immunotech (Westbrook, ME). J18 antiserum, against the major components of 804G matrix was described by Langhofer et al. (1993). The mouse monoclonal antibody 5C5 against the 150 kDa chain of 804G laminin-5 was characterized by Langhofer et al. (1993). An additional mouse monoclonal antibody (CM6) against the same chain of laminin-5 was generated in identical fashion to 5C5 (Langhofer et al., 1993).

Cloning and analysis of the rat laminin-5 α chain cDNA

An 804G lambda zap expression library was produced by Invitrogen Corp., San Diego, CA. and screened with J18 serum (see above), according to the method of Huynh et al. (1985). To characterize positive clones, plaque lifts of nitrocellulose-bound fusion protein were used to epitope select antibodies (Sambrook et al., 1989). In addition, these clones were screened using 5C5 monoclonal antibody. The cDNA insert of one 5C5 reactive clone (150-11), which was also recognized by J18 antibodies, was subcloned into Bluescript (Stratagene, La Jolla, CA) and sequenced by the dideoxy chain termination method (Sanger et al., 1977), using a USB Sequenase kit (USB, Cleveland, OH). This clone was 2.3 kb in length. To obtain additional sequence, 5′ and 3′ RACE (rapid amplification of cDNA) was undertaken. Specifically, poly(A) RNA, generated from 3×10^6 804G cells using a Micro-FastTrack kit (Invitrogen), was used to produce template DNA. This was converted to double stranded cDNA using a Marathon PCR kit (Clonetech) and then 5′ RACE was performed. In this method, specific linkers were ligated to the ends of the double stranded cDNA to allow amplification of the sequence between a linker-specific primer (AP-1, Clonetech) and a gene-specific primer as noted below. In the initial round of amplification, the AP-1 primer (5′-CCATCCTAATAC-GACTCATATAAGGGC-3′) was used with the anti-sense gene-specific primer α-MR (5′-CCAAACTTCAGGACCTGCGGTTC-3′, complementary to nucleotides located towards the 5′ end of clone 150-11). All PCR reactions were carried out in a 50 μl volume containing template DNA, 0.2 mM dNTPs, 0.2 μl AP-1 primer, 0.4 μg gene-specific primer, and 'Expand Long Template PCR' polymerase with 1× buffer #2 (Boehringer Mannheim). Template DNA for primary PCR reactions was diluted according to the Clonetech protocol and 10% of this initial reaction was used in the secondary PCR reactions. Oligos were synthesized on a Cyclone Plus DNA Synthesizer (Millipor) using PerSeptive Biosystems GmbH reagents and the manufacturer's protocols. Reactions were run in a DNA Thermal Cycler (Perkin Elmer) for 30 cycles as follows: 94°C denaturation for 2 minutes, 50°C annealing for 2 minutes and 72°C extension for 3 minutes. This was followed by a 7 minute extension time at 72°C. Appropriate negative controls (lacking primer or template or enzyme) and positive controls (with primers designed to yield fragments of known sizes) were included. PCR fragments were subcloned into the pCR-II vector (Invitrogen) following the manufacturer's protocol. To confirm the identity of PCR products, PCR fragments were subjected to a second round of amplification using AP-1 and a nested gene specific primer.

Methods and template used to clone the 3′ end of the 5C5 antigen were identical to those used to clone the 5′ end except different primers were used. The AP-1 primer was used in conjunction with α-MR2 primer (5′-GAGTCTCAGGAGGCAGTTATGGAC-3′, a region located towards the 3′ end of clone 150-11).

PCR products were subcloned into the vector pCR-II (Invitrogen) and sequenced in both directions by the Sanger method using the Sequenase kit (United States Biochemical). Sequence analyses and secondary structural predictions were made using the GCG sequence analysis software package (University of Wisconsin Biotechnology Center, Madison, WI).

Immunofluorescence and immunoelectron microscopy

Cells to be processed for integrin antibody localization were washed thoroughly in PBS and then fixed for 2 minutes in 3.7% formaldehyde. Cells were subsequently extracted in 0.5% Triton X-100 at 4°C for 8 minutes. For localization of BP230 antigen, cells were extracted in –20°C acetone for 2 minutes and then left to air dry. Double label immunofluorescence was performed as detailed by Riddelle et al. (1991). For visualization of in vivo bound CM6 or 5C5 antibodies, cells were incubated in goat anti-mouse fluorescein-conjugated antibodies. After mounting, coverslips were viewed on a Zeiss LSM10 confocal microscope (Carl Zeiss, Thornwood, NY).

For immunogold localization of antibody treated cell preparations, cells on glass coverslips were extracted in –20°C acetone for 2 minutes and then air dried. The cells were overlaid with BP230 antibody and incubated at 4°C overnight. Following extensive washing, the preparations were incubated for 6 hours at room temperature in a mixture of 5 nm gold-conjugated goat anti-mouse IgG and 10 nm gold-conjugated goat anti-human IgG (Amersham Corp., Arlington Heights, IL). The cells were washed, fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and processed for electron microscopical examination as detailed below.

Controls for our immunocytochemical studies included omission of primary antibodies or use of irrelevant IgG to determine non-specific binding of secondary antibodies. In addition controls for double labels included incubation with single primary antibody, followed by incubation with a mixture of conjugated secondary antibodies e.g. mouse primary followed by a mix of fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-human IgG. This allowed us to assess inappropriate cross reactivity of secondary antibodies.

Isolation of laminin-5 from 804G conditioned medium

Laminin-5 was prepared from the conditioned medium of 804G cells (Hormia et al. 1995). In brief, 804G spent medium is fractionated by cation exchange chromatography. Fractions enriched in 5C5 antigen are further processed by anion exchange chromatography and a final purification is achieved using hydroxyapatite chromatography. This procedure will be detailed elsewhere (M. Fitchmun et al., manuscript in preparation).

Gel electrophoresis and immunoblotting

804G matrix was solubilized in a buffer containing 8 M urea, 1% SDS, 10 mM Tris-HCl, pH 6.8, and 15% β-mercaptoethanol (sample buffer). To prepare whole cell extracts, confluent dishes of 804G cells were washed in PBS and then cells were solubilized in sample buffer. To disrupt DNA, each extract was sonicated using a 50 W Ultrasonic Processor (Vibracell Sonics and Materials Inc., Danbury, CT). Protein samples were subjected to SDS-PAGE using 6% acrylamide gels. Two-dimensional gels were prepared according to the method of Hunzicker-Dunn et al. (1991) using pH 3-10 ampholines. Proteins separated on 1- and 2-dimensional gels were transferred to nitrocellulose for immunoblot analyses. Immunoblots were developed using a LumiGLO chemiluminescent substrate kit (Kirkegaard and Perry Labs., Gaithersburg, MD).

Electron microscopy

Fixing and processing of cells for electron microscopy was performed as described by Riddelle et al. (1991). Sections were cut perpendicular to the substratum and viewed on a JEOL 100CX electron microscope at 60 kV.

Laminin-5 was diluted to a final concentration of 5 μg/ml with 3 mM formic acid containing 70% glycerol. For antibody localization, 1 μg/ml of antibody was added to 50 μg/ml of laminin-5 in a total volume of 25 μl. The mix was incubated at room temperature for 2 hours and then diluted 10-fold with the formic acid, glycerol mix. A 5 μl sample of the protein was then sandwiched between two pieces of freshly cleaved mica. The mica pieces were separated and then mounted on a rotary stage in a Balzers freeze etch unit (model BAF400T, Balzers AG, Lichtenstein), dried under vacuum, and then coated with a platinum/carbon film at a low angle (4°) to an approximate depth of 27 nm. The platinum/carbon was then stabilized by coating with a carbon film at 90°. The sample was removed from the unit and replicas were placed on electron microscope grids. The grids were viewed on a JEOL 100CX electron microscope at 60 kV.

RESULTS

5C5 and CM6 antibodies both recognize the 150 kDa α chain of rat laminin-5

We have prepared a panel of antibodies against the major components of the matrix secreted by 804G cells (Langhofer et al., 1993; G. Plopper, unpublished results). Two of these antibodies, 5C5 and CM6, recognize a 150 kDa polypeptide in 804G matrix (Fig. 1; Langhofer et al., 1993; Jones et al., 1994). To confirm that 5C5 and CM6 antibodies recognize the same 804G matrix species, we processed 804G matrix for 2-dimensional gel electrophoresis followed by western immunoblotting

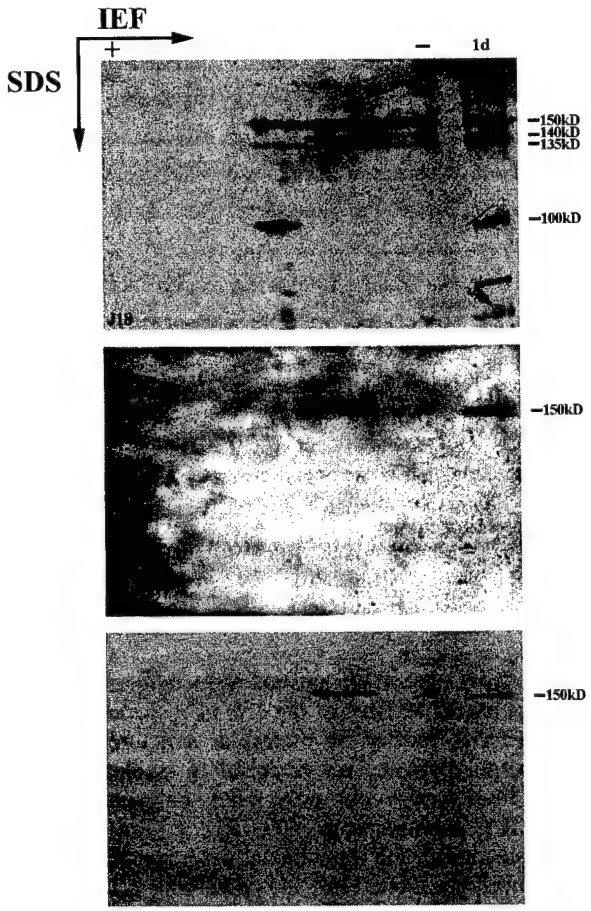


Fig. 1. 804G matrix was processed for 1- and 2-dimensional gel electrophoresis and then separated polypeptides were transferred to nitrocellulose. The resulting nitrocellulose sheets were incubated in either J18 serum, CM6 or 5C5 antibodies. J18 antibodies recognize polypeptides of 150, 140, 135 and 100 kDa in the 1-dimensional (1d) profile. The 150, 140 and 135 kDa proteins each resolve into several, primarily basic, variants in the 2-dimensional immunoblot. CM6 and 5C5 antibodies recognize a 150 kDa protein in 1-dimensional immunoblots and show identical staining of variants of this 150 kDa polypeptide in the second dimension. IEF, isoelectric focusing.

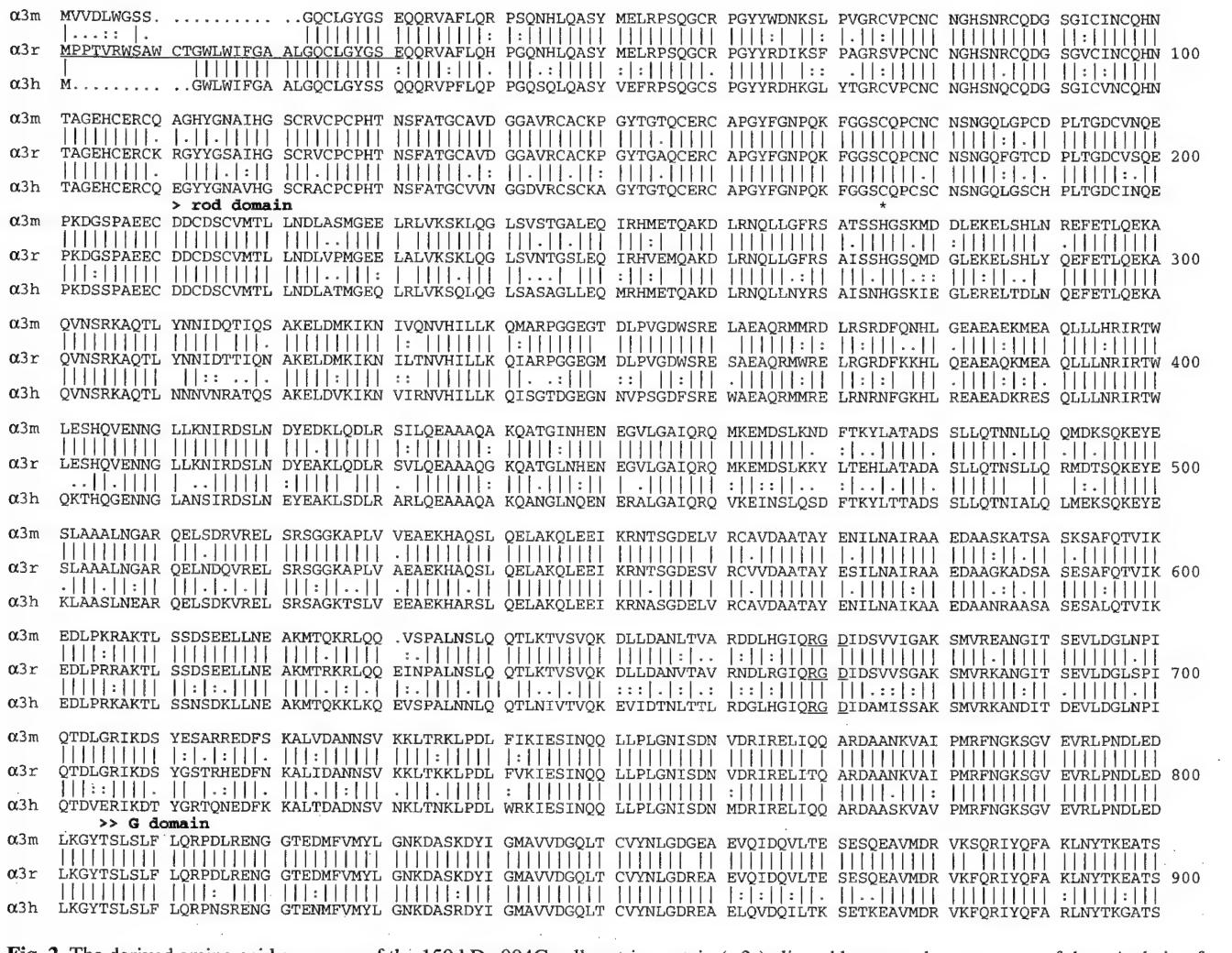


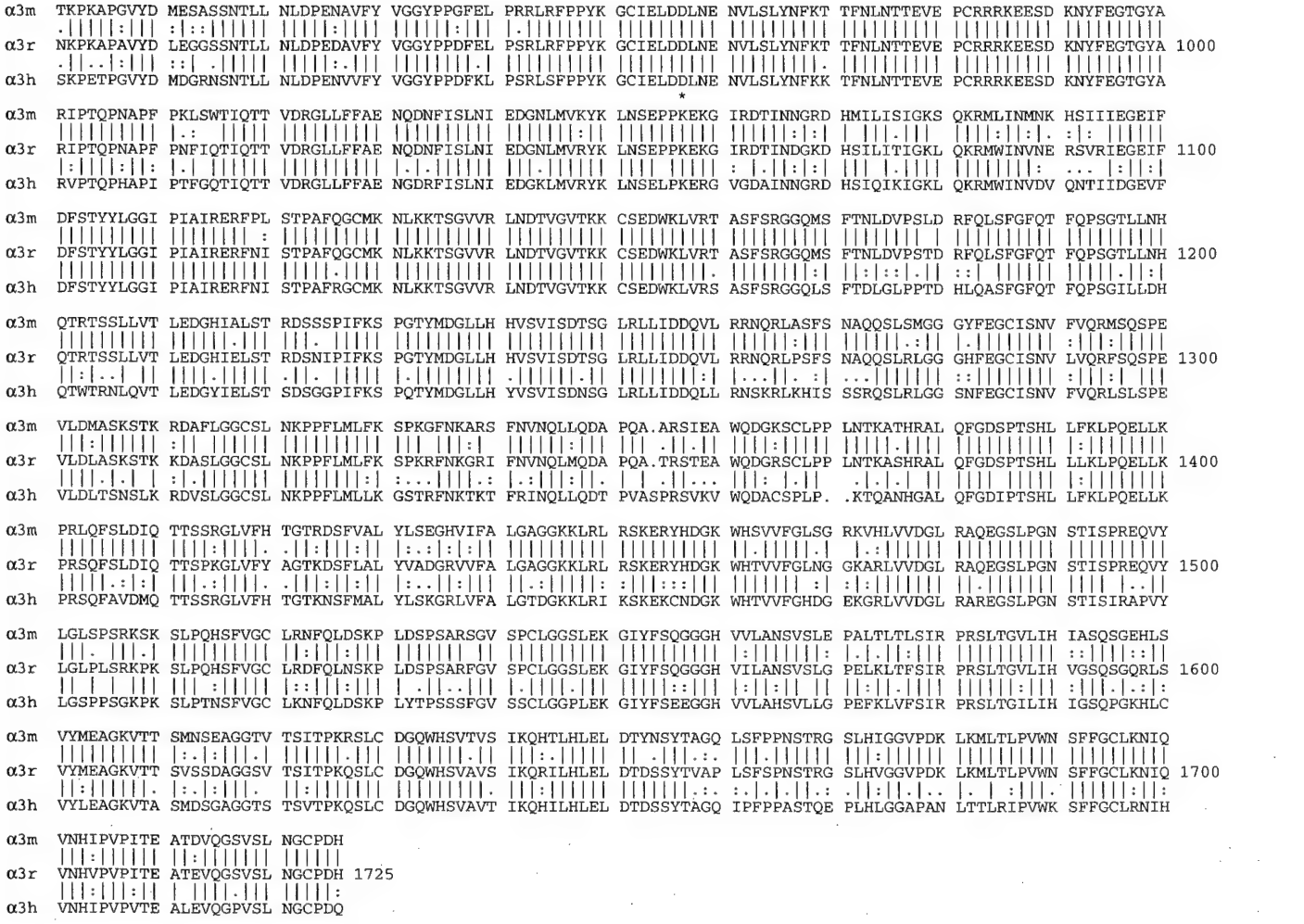
Fig. 2. The derived amino acid sequence of the 150 kDa 804G cell matrix protein (α3r) aligned between the sequences of the αA chain of nicein (α3m) and the αA chain of epiligrin (α3h). The sequences were aligned using the sequence analysis software package of the Genetics Computer Group (GCG). Amino acids in α3m and α3h that showed identity with amino acids in α3r are indicted by vertical dashes. Colons represent conservative changes while single dots indicate less conservative differences. An RGD adhesion recognition site common to α3m, α3r and α3h is underlined as is the putative signal peptide of α3r (labels are placed above the start residue of the corresponding domain). The rod and G domains are indicated. The start and end of the deduced amino acid sequence of clone 150-11 are marked by asterisks. The α3r sequence has been submitted to GenBank (accession number U61261).

(Fig. 1). Both 5C5 and CM6 antibodies recognize an identical series of isoelectric variants of the 150 kDa species (Fig. 1).

To characterize further the molecular structure of the 150 kDa matrix polypeptide, we screened a lambda-zap 804G expression library with our polyclonal, polyspecific matrix antiserum J18 (Langhofer et al., 1993; Fig. 1). Positive plaques were rescreened with the monoclonal antibody 5C5, resulting in the isolation of a clone (150-11) containing a 2.3 kb insert. The fusion protein expressed by this cDNA is recognized in western blots by 5C5 antibodies (not shown). In addition, the recombinant protein produced by clone 150-11 was adsorbed onto filters and used to immunoselect antibodies from the J18 antiserum. These immunoselected antibodies recognize the 150 kDa 804G matrix polypeptide by immunoblotting (not shown).

Analyses of the nucleotide sequence of clone 150-11 identified an open reading frame spanning the entire 2.3 kb insert (Fig. 2). Because of the lack of 5′ or 3′ untranslated regions, as well as the lack of translational start or stop codons, this clone likely represented a partial fragment of a larger mRNA. This was confirmed by northern blotting which showed that the message encoding the 5C5 antigen was approximately 5.5 kb (result not shown). To complete the sequence of the 5C5 antigen we have performed 5′ and 3′ RACE as detailed in Materials and Methods. The sequence consists of an open reading frame of 5,175 bp with an additional 81 bp of 5′ and 29 bp of 3′ untranslated region (result not shown). The deduced amino acid sequence of the completed sequence is shown in Fig. 2. An homology search in the GenBank reveals that the latter shows 76% identity to the α3A type chain of the human basement membrane protein complex called epiligrin/kalinin/laminin-5, and 88% identity to the sequence of the α3A type chain of mouse nicein, the mouse homologue of epiligrin/kalinin/laminin-5 (Ryan et al., 1994; Aberdam et al., 1994a; Burgeson et al., 1994; Galliano et al., 1995; Fig. 2).

The degree of similarity between the deduced sequence of



the 150 kDa chain of 804G matrix with the α chain of mouse and human laminin-5 provides evidence that this polypeptide is the rat homologue of the α chain of laminin-5. This result, together with previous data indicating that the 140 kDa protein component of 804G matrix species is a γ2 laminin chain, suggests that the major structural element of 804G matrix is rat laminin-5 (Langhofer et al., 1993).

The α3 chain of 804G matrix consists of 1,725 residues with a predicted molecular mass of 190 kDa. This is 40 kDa larger than the major species recognized by 5C5 (and CM6) antibodies in immunoblots (Fig. 1). Indeed, we speculate that the rat 150 kDa 5C5 antigen undergoes proteolytic processing. This would be consistent with evidence that the α3A chain of human laminin-5 is cleaved extracellularly from 200 kDa to approximately 165 kDa (Marinkovich et al., 1992; Ryan et al., 1994). The carboxy terminal G domain of rat α3 comprises 921 amino acids which is separated by a rod-like region of 591 residues from a short arm (Fig. 2). A potential cell adhesion (RGD) sequence matches those in the mouse and human α3 chains (Fig. 2). There is a putative Met start codon and a signal peptide sequence with a consensus cleavage site at Glu residue 31 in α3 (Von Heijne, 1986; Fig. 2). There is some divergence in the amino terminal sequence of rat α3 compared with the corresponding mouse and human chains (Fig. 2).

CM6 and 5C5 antibodies bind distinct domains in the laminin-5 molecule

Laminin-5, purified from 804G conditioned medium, consists of three major polypeptides of 150, 140 and 135 kDa (Fig. 3A; Hormia et al., 1995). The same preparation was viewed in the electron microscope following rotary shadowing. The laminin-5 molecules appear predominantly as Y-shaped structures, each possessing a 'long' arm of approximately 110 nm in length (Fig. 3B). Such stuctures are consistent with images of human laminin-5 presented by Rousselle et al. (1991). As is the case for other laminins, the end of the long arm of the laminin-5 molecule is globular (Fig. 3B; panels 1-5). This is commonly referred to as the G domain (Tryggvason, 1993). To define better the CM6 and 5C5 antibody epitopes, laminin-5 was incubated for 2 hours at room temperature with each antibody. The laminin-5/antibody mix was then processed for rotary shadowing. In the case of the CM6 antibody treated sample, 30 out of 49 molecules show two obvious globules at the end of the long arm, furthest from the fork in the Y structured molecule, indicating that the CM6 antibodies bind the G domain. We show four examples (Fig. 3B, panels 6-9). In contrast, in the absence of antibody, 64 out of 64 laminin-5 molecules show only a single globule at the end of their long arms (Fig. 3B, panels 1-5). 5C5 antibodies bind the long arm of laminin-5 towards the fork in the Y structure of the molecule

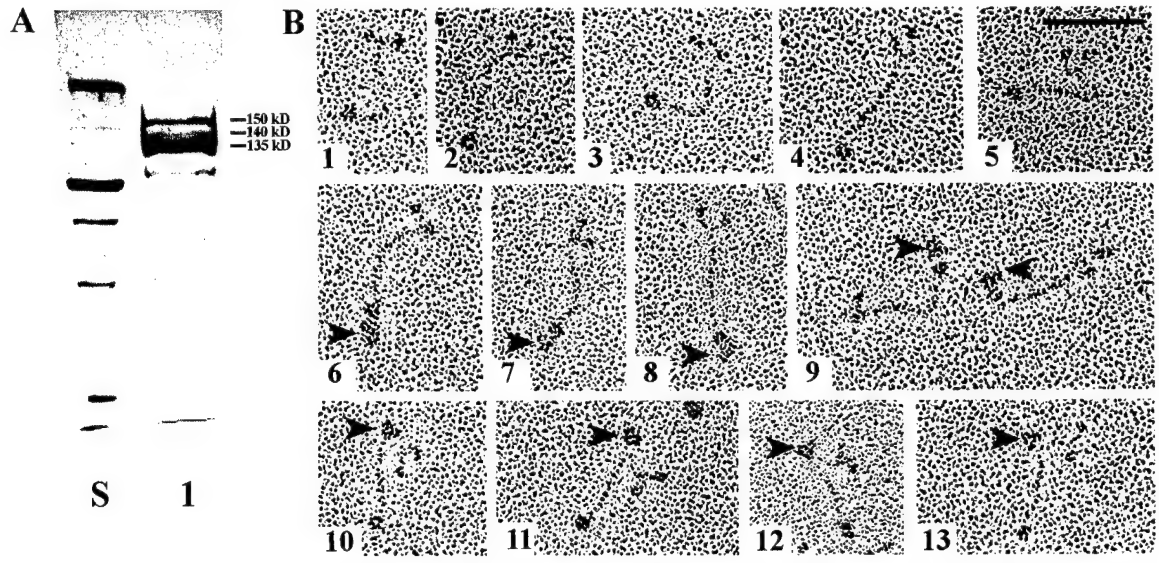


Fig. 3. In A, in lane 1, 5 μg of column purified laminin-5 was processed for SDS-PAGE. Note that laminin-5 is composed of three major polypeptides of 150, 140 and 135 kDa. One minor protein of around 120 kDa is also present in the preparation. Molecular mass standards in lane S are (from top to bottom) 200, 116, 97, 66 and 45 kDa. In B, laminin-5 was prepared for rotary shadowing electron microscopy alone (1-5), or following incubation with CM6 (6-9) or 5C5 antibodies (10-13). Note that CM6 antibodies bind the large globular end (G domain) of the Y-shaped laminin-5 molecule (arrowheads in 6-9). 5C5 antibodies bind close to the neck of the Y-shaped molecule (arrowheads in 10-13). Bar, 100 nm.

(Fig. 3B, panels 10-13). The 5C5 antibodies apparently induce a kink in laminin-5 (Fig. 3B, panels 10-13).

CM6 and 5C5 localization in 804G cells

Both 5C5 and CM6 localize in a 'leopard spot' pattern along the substratum attached surfaces of 804G cells processed for immunofluorescence and viewed by confocal laser scanning microscopy (Langhofer et al., 1993; Fig. 4). Furthermore, in 804G cells processed for double label immunofluorescence, 5E antibody staining localizes at sites where there are concentrations of CM6 antigen (Fig. 4).

CM6 antibodies disrupt hemidesmosomes in 804G cells

We have assayed the impact of our matrix antibodies on formed hemidesmosomes. For these studies, 804G cells were plated and 48 hours later various matrix antibodies were added at various concentrations ranging from 10-100 μg/ml to their medium. After 2 hours, the treated 804G cells were prepared either for immunofluorescence or electron microscopy. In cells incubated with 5C5 antibodies at concentrations of 10-100 μg/ml and then processed for immunofluorescence microscopy, the α6 integrin subunit and BP230 co-localize with in vivo bound 5C5 antibodies (Figs 5 and 6). It should be noted that there are some areas of 5C5 antibody staining where there is no obvious corresponding BP230 and α6 integrin staining. However, there is no BP230 and α6 integrin staining without corresponding 5C5 antibody matrix staining (Figs 5 and 6). Ultrastructural examination of the cells reveals that they possess many normal appearing hemidesmosomes at sites of cell-substrate interaction (not shown). Because 5C5 antibodies have no obvious impact on hemidesmosomes or the localization of hemidesmosmal antigens in 804G cells, we use 5C5 antibodies as a control in all of our subsequent studies where we analyze in vivo effects of our second α chain antibody CM6.

When 804G cells which had been incubated with CM6 antibodies at concentrations as low as 50 μg/ml, are prepared for immunofluorescence, α6 integrin and BP230 fail to co-localize with the in vivo bound CM6 antibodies (Figs 5 and 6). For this and subsequent studies we show results for experiments where we use 50 μg/ml of CM6 antibodies. Like the 5C5 antibodies, CM6 antibody is able to bind its antigen in living cells and stains in a leopard spot pattern (Figs 5E and 6E). In contrast, both α6 and BP230 stain primarily cytoplasmic aggregates

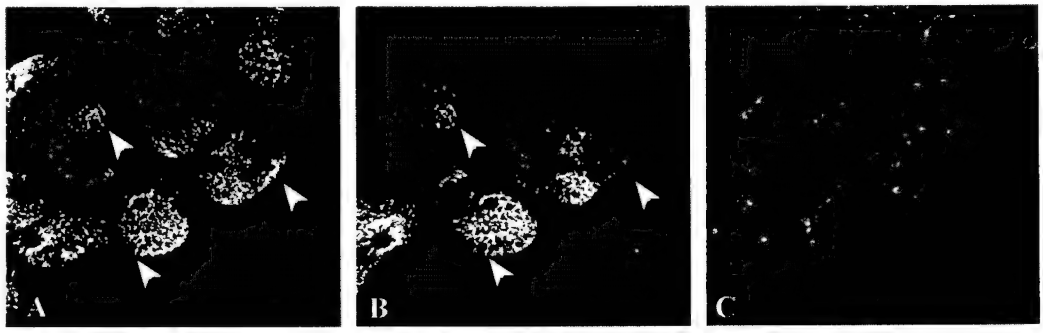


Fig. 4. 804G cells were processed for double label immunofluorescence using the human monoclonal antibody, 5E, which recognizes BP230 (A) and CM6 mouse monoclonal antibody against the α3 chain of laminin-5 (B). 5E antibodies (A) localize in a pattern which 'mirrors' the organization of the underlying matrix as stained by CM6 antibodies (B)(arrowheads). (C) Phase contrast. Bar, 25 μm.

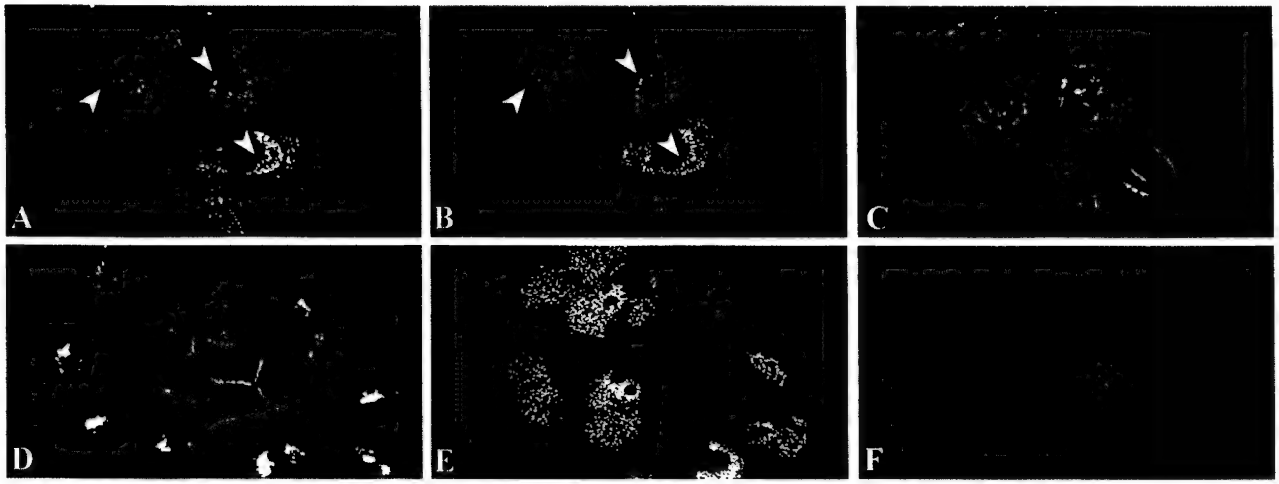


Fig. 5. 804G cells were plated onto glass coverslips; 48 hours later either 5C5 (A-C) or CM6 monoclonal antibodies (D-F) at 50 μg/ml were added to the cell medium. After a 2 hour incubation, the cells were processed for immunofluorescence microscopy with affinity purified α6 polyclonal antibodies (A,D). To visualize in vivo bound 5C5 and CM6 antibodies, the preparations were treated with fluorescein-conjugated goat anti-mouse IgG (B,E) while the α6 antibodies were visualized using rhodamine-conjugated goat anti-rabbit IgG (A,D). Note that the α6 integrin subunit (A) localizes in a pattern which 'mirrors' the underlying matrix stained by 5C5 antibodies (B)(arrowheads). In contrast, in CM6 antibody treated 804G cells, α6 integrin staining is concentrated in cytoplasmic aggregates or at areas of cell-cell contact (D) and shows no obvious co-localization with the in vivo bound CM6 antibodies, which are distributed in a leopard spot pattern along the cell-substratum interface (E). (C,F) Phase contrast. Bar, 25 μm.

(Figs 5D and 6D). Additionally, in the case of the α6 integrin subunit, there is some cell-cell staining which we do not observe in control or 5C5 antibody treated cells (Fig. 5D).

In thin sections of 804G cells incubated with CM6 antibodies viewed in the electron microscope, various stages of internalization of hemidesmosomes can be observed (Fig. 7). In some cells, hemidesmosomes clearly become detached from the substrate (Fig. 7A and B). Intriguingly, entire hemidesmosome complexes including the extracellular anchoring filaments appear to become internalized in large vesicles (Fig. 7C). On the outside or cytoplasmic face of each vesicle, hemidesmosome cytoplasmic plaques are evident,

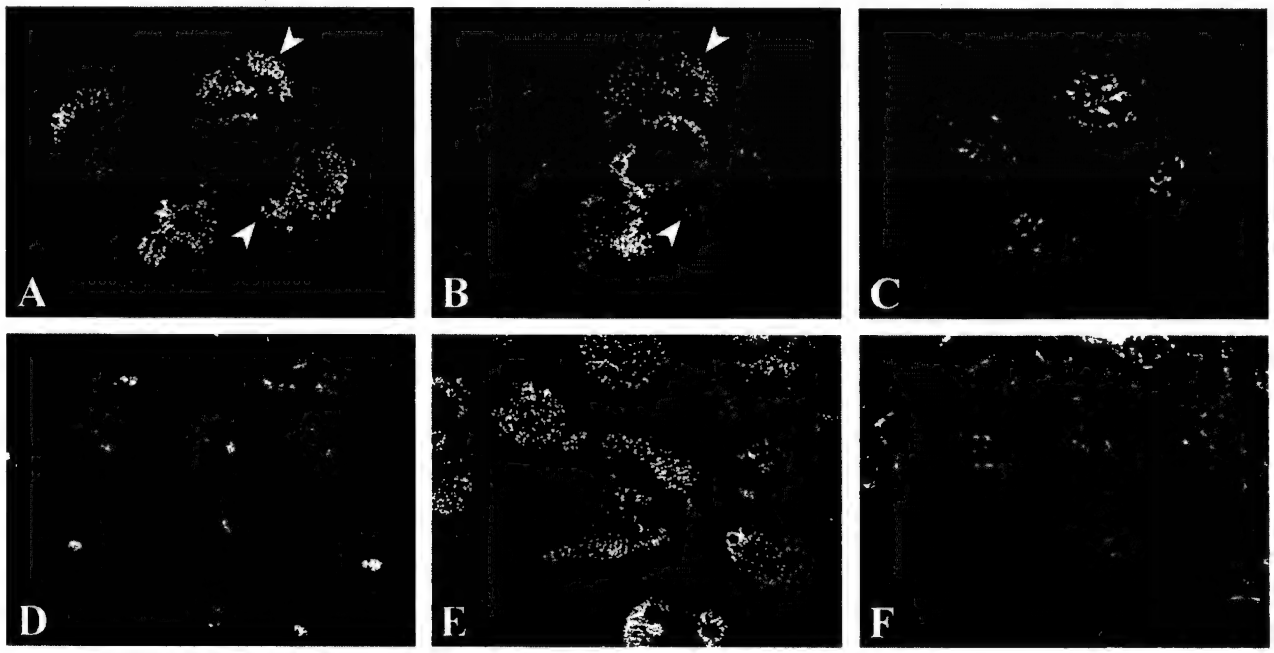


Fig. 6. 804G cells were plated onto glass coverslips; 48 hours later either 5C5 (A-C) or CM6 monoclonal antibodies (D-F) at 50 μg/ml were added to the cell medium. After a 2 hour incubation, the cells were processed for immunofluorescence microscopy using 5E antibodies against BP230 (A,D). Localization of in vivo bound 5C5 or CM6 antibodies is shown in B and E, respectively. In 5C5 antibody treated 804G cells, most, if not all, 5E antibody staining in A is found colocalized with 5C5 antigen (B)(arrowheads) at the cell-substratum interface. However, in 804G cells treated with CM6 antibodies, BP230 localizes primarily in cytoplasmic aggregates (D), while CM6 antibodies show a leopard spot pattern (E) along the substrate of the cells. (C,F) Phase contrast. Bar, 25 μm.

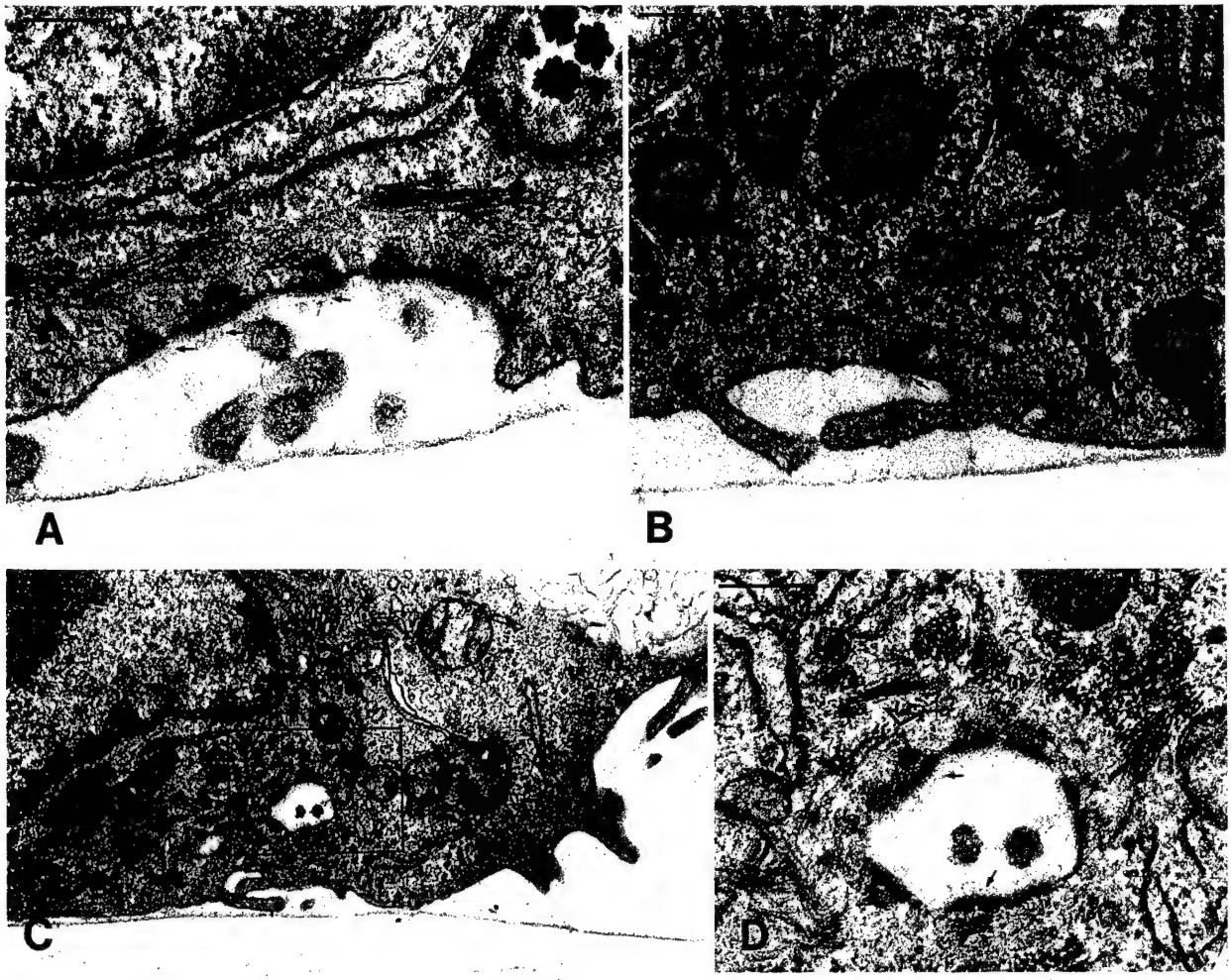


Fig. 7. 804G cells were plated onto glass coverslips; 48 hours later CM6 monoclonal antibodies at 50 μg/ml were added to the cell medium. After a 2 hour incubation, the cells were processed for electron microscopy. (A-D) Hemidesmosomes in various stages of being internalized. (A) A group of hemidesmosomes have detached from the cell substrate. Anchoring filaments remain associated with the membrane of each hemidesmosome (A, arrows). (B) A detached hemidesmosome in an early stage of internalization (the arrow in B indicates anchoring filament-like structures). (C) A low power view of a cytoplasmic vesicle with several associated hemidesmosomes (boxed area). (D) A higher power view of the boxed area showing that hemidesmosomes line the vesicle. Each hemidesmosome has an obvious cytoplasmic plaque which is associated with keratin bundles (open arrow). Anchoring filament-like structures of each hemidesmosome extend inside the vesicle (arrows). Bars: (A,B,D), 200 nm; (C), 500 nm.

each retaining its interaction with keratin filaments (Fig. 7D). Anchoring filaments and sub-basal dense plates line the inside of the vesicles (Fig. 7D). To extend these observations, antibody treated cells were prepared for immunogold localization. At 2 hours following 5C5 treatment, cells have normal appearing hemidesmosomes which are recognized by BP230 antibodies (Fig. 8A). 5C5 antibodies localize extracellularly, underlying each hemidesmosome (Fig. 8A). In CM6 treated 804G cells, 10 nm gold particles, indicating BP230 localization, are found in association with the internalized hemidesmosome structures (Fig. 8B). Moreover, CM6 antibodies are clustered extracellularly along the cell-substrate interface (Fig. 8B).

To ensure that the impact of CM6 antibodies on 804G cells is reversible, we trypsinized the CM6 antibody treated 804G cells and then plated the detached cells into fresh medium. Over 90% of the antibody treated cells attach to the new growth substrate. Furthermore, the latter cells all express hemidesmosomal antigens, which were organized in a leopard spot pattern at the base of the cells, and assemble hemidesmosomes (results not shown).

We next studied the impact of CM6 antibodies on de novo hemidesmosome assembly using a model system described by Langhofer et al. (1993). In this system, keratinocytes are induced to assemble hemidesmosomes by 804G matrix. We therefore assessed the impact of CM6 and 5C5 antibodies on adhesion of SCC12 keratinocytes to 804G matrix via hemidesmosomes.

CM6 antibodies block influence of 804G matrix on hemidesmosome protein localization and hemidesmosome assembly

In these studies, we have been able to monitor the impact of CM6 antibodies on the ability of 804G matrix elements to influence the organization of hemidesmosomal proteins along the cell-matrix interface in SCC12 cells, a process described by Langhofer et al. (1993).

SCC12 cells were plated onto CM6 or 5C5 antibody treated

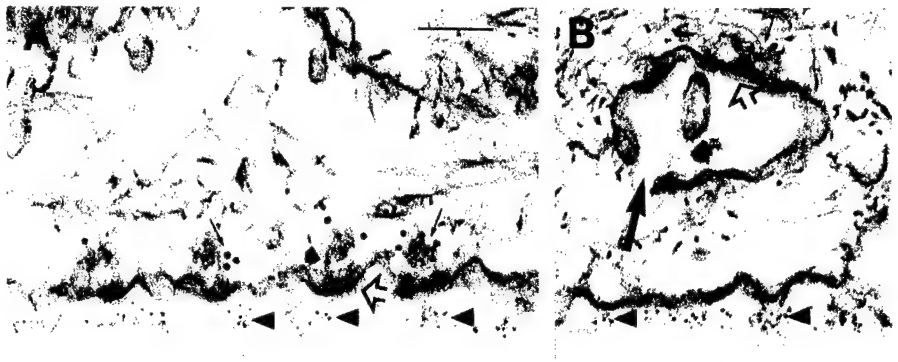


Fig. 8. At 48 hours after plating, 804G cells were treated for 2 hours either with 50 μg/ml of 5C5 (A) or CM6 (B) antibodies. The cells were subsequently processed for immunoelectron microscopy using human monoclonal antibody, 5E, against BP230. The latter were detected using 10 nm gold-conjugated goat anti-human IgG secondary antibodies. In vivo bound 5C5 and CM6 were detected using 5 nm gold-conjugated anti-mouse secondary antibodies. In A, 10 nm gold particles (arrows) are clustered on the cytoplasmic plaques of several hemidesmosomes located at the cell-substrate interface. Underlying each hemidesmosome is a group of 5 nm gold particles (arrowheads). The open arrow marks the sub-basal dense plate of a hemidesmosome. In B, a vesicle is indicated by the large arrow. On one side of this vesicle is a hemidesmosome-like structure (small arrow); 10 nm gold paticles are associated with this structure and an open arrow marks a sub-basal dense plate. Along the cell-substrate interface in B there are numerous 5 nm gold particles (arrowheads). Bar, 100 nm.

804G matrix for 4 hours and then processed for immunofluorescence. To confirm that 5C5 and CM6 antibodies bound matrix, the preparations were incubated in fluorochrome-conjugated anti-mouse IgG. The α6 integrin subunit and BP230 protein in SCC12 cells were detected using GoH3 and 5E antibodies, respectively. In SCC12 cells maintained on matrix pre-incubated with 5C5 antibodies, both α6 and BP230 antigens concentrate at sites where in vivo bound 5C5 antibodies localize in a characteristic leopard spot pattern. There are some areas of 5C5 antibody staining without corresponding BP230 and α6 integrin reactivity. In contrast, no areas of BP230 and α6 integrin staining are observed without co-localizing 5C5 antibody stained matrix (Figs 9 and 10). In cells plated onto CM6 treated matrix, neither the α6 integrin subunit nor BP230 show obvious colocalization with the in vivo bound CM6 antibodies. Rather, both BP230 and α6 integrin show localization in streaks and rows of spots towards the cell periphery while the CM6 antibodies show the same sort of leopard spot pattern as 5C5 (Figs 9 and 10). The pattern of

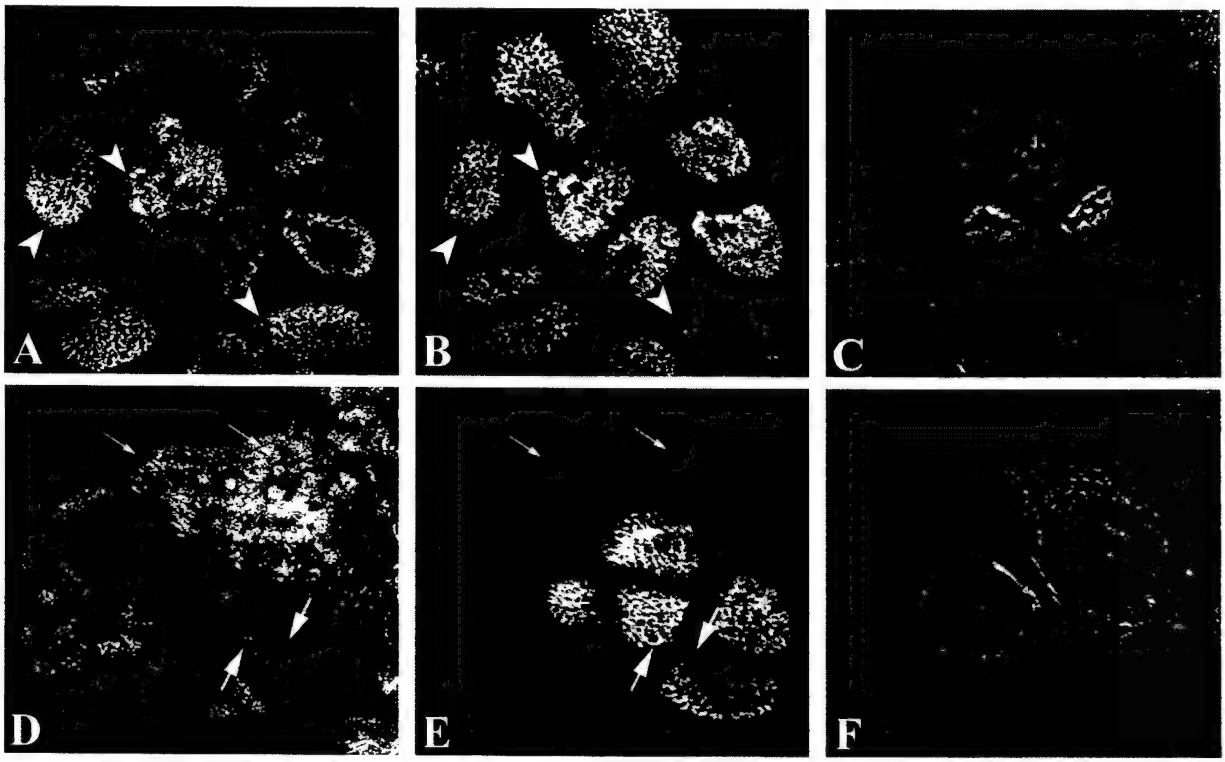


Fig. 9. SCC12 cells maintained on 5C5 (A-C) or CM6 (D-F) antibody treated 804G matrix for 4 hours were processed for immunofluorescence microscopy. In vivo bound antibodies were visualized in B and E using fluorochrome-conjugated goat anti-mouse IgG. Affinity purified antibodies to the α6 (A) integrin subunit stain in a pattern very similar to the underlying matrix elements recognized by 5C5 antibodies in B (arrowheads). In contrast, α6 staining (D) of SCC12 cells on CM6 antibody treated matrix, is localized in streaks or patches at the basal aspect of the cell and fails to show any obvious colocalization with the in vivo bound CM6 antibodies (E). (D and E) Small arrows denote areas where there is α6 staining in the absence of matrix staining, while large arrows indicate areas of CM6 antibody localization but no α6 integrin. (C,F) Phase contrast. Bar, 25 μm.

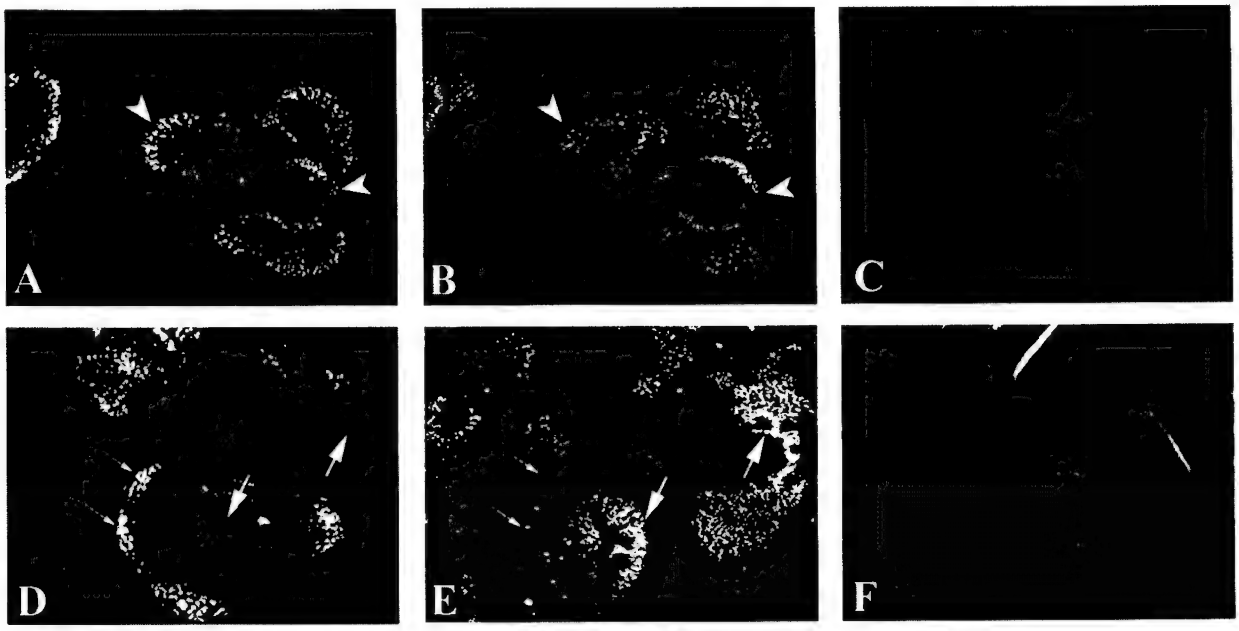


Fig. 10. SCC12 cells maintained on 5C5 (A-C) or CM6 (D-F) antibody treated 804G matrix for 4 hours were processed for immunofluorescence microscopy using human monoclonal antibody, 5E, against BP230 (A,D). In vivo bound 5C5 and CM6 antibodies were visualized in B and E using fluorochrome-conjugated goat anti-mouse IgG. In cells maintained on 5C5 antibody treated 804G matrix, BP230 staining in A clearly localizes with underlying matrix stained by 5C5 antibodies (B)(arrowheads). However, in cells plated onto CM6 treated 804G matrix, BP230 staining (D) fails to colocalize with the pattern of staining generated by CM6 antibodies (E). Rather BP230 is concentrated at cell edges. Small arrows in D and E indicate areas of BP230 antibody staining but no CM6 antibody localization while large arrows indicate the converse. (C,F) Phase contrast. Bar, 25 μm.

BP230 and α6 integrin in the latter is identical to that observed if the cells are maintained on rat tail collagen or glass as reported by Langhofer et al. (1993). In other words, CM6 antibodies block matrix-determined organization of hemidesmosomal antigens along regions of SCC12 cell-804G matrix interaction.

At the ultrastructural level, 804G matrix induces assembly of hemidesmosomes in SCC12 cells (Langhofer et al., 1993). Thus to extend the immunofluorescence observations detailed above, SCC12 cells plated onto CM6 or 5C5 antibody treated 804G matrix were also processed for electron microscopy at 4 hours after plating. SCC12 cells plated onto the 5C5 antibody treated matrix assemble hemidesmosomal structures at sites of cell-substrate interaction (Fig. 11). These hemidesmosomes consist of an electron dense cytoplasmic plaque interacting with IF as well as anchoring filaments and a sub-basal dense plate. In contrast, we have observed no obvious hemidesmosomes in SCC12 cells plated on the CM6 antibody treated matrix (Fig. 11).

DISCUSSION

In this study we have used an immunological approach to show that laminin-5 plays an essential role in nucleation of hemidesmosome assembly. More specifically, our data reveal that the α chain of laminin-5, secreted by 804G cells, contains an epitope, recognized by CM6 but not by 5C5 antibodies, which is involved not only in induction of hemidesmosome assembly but which is also necessary for maintaining the structural integrity of formed hemidesmosomes. Intriguingly, we provide evidence that the α chain epitope recognized by CM6 antibodies is located within the G domain of the laminin-5 molecule. This finding has intriguing parallels with what is known about the cell binding domains of other laminins. For example, many cellular interactions of laminin-1 are mediated by the G domain of its α chain (Skubitz et al., 1991; Yurchenco et al., 1993; Matter and Laurie, 1994).

It is becoming clear that laminin-5 is a promiscuous matrix ligand. Carter et al. (1991) have shown that α3β1 integrin is involved in early stage keratinocyte adhesion to epiligrin (another term for laminin-5). In contrast, in certain other cells, including OVCAR-4 and HBL100, α6β1 integrin acts as a receptor for laminin-5 (Rousselle and Aumailley, 1994). Laminin-5 is spatially associated with α6β4 integrin in formed hemidesmosomes (Rousselle et al., 1991; Jones et al., 1994). Indeed, Niessen et al. (1994) and Spinardi et al. (1995) have provided experimental evidence for direct binding of α6β4 to laminin-5. The latter studies, taken together with those reported by Jones et al. (1991) and Kurpakus et al. (1991), support the idea that assembly of hemidesmosomes is nucleated upon interaction between laminin-5 and the α6β4 integrin receptors of an epithelial cell. Data here suggest that CM6 antibodies block this interaction, either by competing for the α6β4 binding site on the α chain of laminin-5 or, in some way, modulating this interaction.

Laminin-5 is a relatively newly identified component of basement membrane (Verrando et al., 1987; Rousselle et al., 1991; Carter et al., 1991; Langhofer et al., 1993; Jones et al., 1994; Burgeson et al., 1994). Recently, analyses of several different blistering diseases have suggested an important role for laminin-5 in mediating epidermal cell anchorage to the basement membrane. For example, mutations in all three laminin-5 subunits (α3, β3 and γ2 chains) have been linked to the dysadhesion of epidermal cells that characterizes Herlitz's junctional epidermolysis bullosa (Pulkkinen et al., 1994a,b; Aberdam et al., 1994b; Kivirikko et al.,

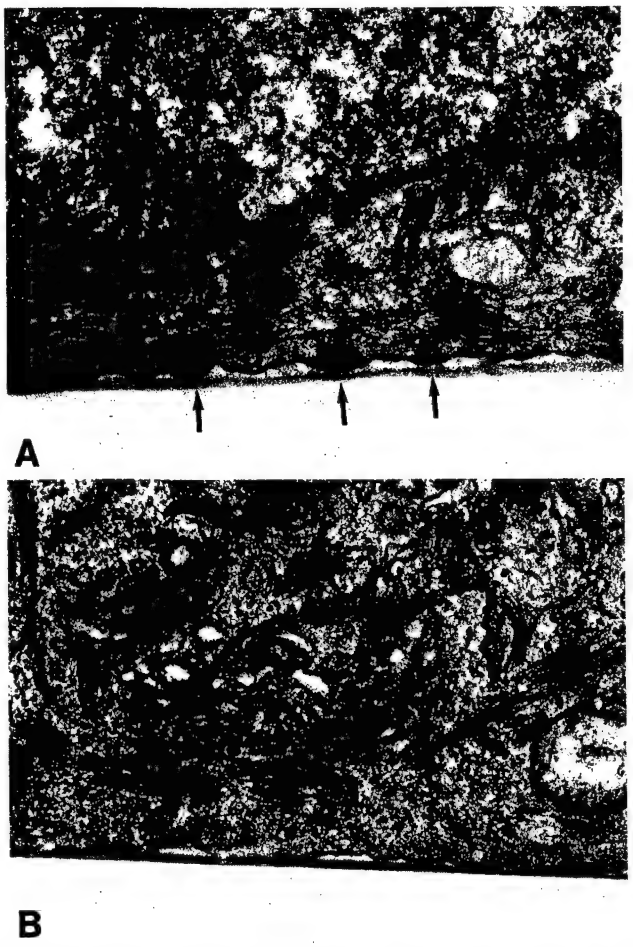


Fig. 11. SCC12 cells cultured on 5C5 or CM6 antibody treated 804G matrix were assayed for assembly of hemidesmosomes by electron microscopy. In SCC12 cells maintained on 5C5 antibody incubated matrix, arrows indicate typical IF associated hemidesmosomal plaques with sub-basal dense plates (A). In contrast, SCC12 cells cultured on CM6 antibody treated 804G matrix do not possess any obvious hemidesmosomes (B). Bar, 200 nm.

1995). Based on the studies we present here, we predict such mutations impact the ability of an epidermal cell to make hemidesmosomes because, for example, they result in an absence of extracellular laminin-5 or in assembly of a laminin-5 molecule in which important functional domains such as the CM6 epitope are unavailable for cell binding. These ideas are consistent with the ultrastructural characteristics of junctional epidermolysis bullosa. That is, skin specimens of affected patients show either a lack of hemidesmosomes or greatly decreased hemidesmosome numbers (Eady et al., 1994; Kivirikko et al., 1995). Indeed, we speculate that a loss of hemidesmosomes, in part, explains the weakening of epithelial cell anchorage to basement membrane that results in blister formation in junctional epidermolysis bullosa.

In addition to being involved in genetic diseases, laminin-5 also appears to be a target in at least one autoimmune disease. In particular, Domloge-Hultsch et al. (1992) have identified circulating laminin-5 autoantibodies in the serum of patients with an acquired mucosal subepidermal autoimmune blistering disease called cicatricial pemphigoid (CP). In this disease, autoantibodies are thought to perturb epithelial cell adhesion to the basement membrane. Such autoantibodies may act by disrupting hemidesmosomes in much the same manner as our CM6 antibodies disrupt formed hemidesmosomes in 804G cells. Whether this mechanism can explain CP blister formation will require more detailed analyses of the pathogenic autoantibodies in CP serum samples.

Laminin-5 is considered to be a major component of anchoring filaments. Immunogold localization reveals that laminin-5 antibodies bind to the lamina lucida side of the lamina densa section of the basement membrane (Rousselle et al., 1991; Jones et al., 1994). We were therefore surprised to observe that both the detached and internalized hemidesmosomes in CM6 antibody treated 804G cells retain anchoring filament-like structures while laminin-5 remains associated with the substrate as shown, ultrastructurally, by the CM6 in vivo bound antibody stain. This result raises some questions as to the exact nature of anchoring filaments and appears consistent with recent speculation that the collagenous domains of BP180 assemble into anchoring filaments (Jones et al., 1994).

In summary, we present direct experimental evidence for the role of laminin-5 in the establishment and maintenance of hemidesmosomes. The hemidesmosome provides a structural link between extracellular matrix and the cytoskeleton of an epithelial cell. Now that molecular interactions between hemidesmosomal components are being defined, we are in a position to assess the intriguing possibility that the hemidesmosome not only functions in adhesion but may also be involved in matrix-cell signaling events (Mainiero et al., 1995). Indeed, in this study we provide support for this; laminin-5 carries a structural cue, defined by the CM6 antibody epitope, which when 'transduced' to the overlying epithelial cells triggers the assembly of a complex multiprotein structure, the hemidesmosome.

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Desmosomes and hemidesmosomes: structure and function of molecular components

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ABSTRACT **Desmosomes and hemidesmosomes are the major cell surface attachment sites for intermediate filaments at cell-cell and cell-substrate contacts, respectively. The transmembrane molecules of the desmosome belong to the cadherin family of calcium-dependent adhesion molecules, whereas those in the hemidesmosome include the integrin class of cell matrix receptors. In each junction, the cytoplasmic domains of certain transmembrane junction components contain unusually long carboxy-terminal tails not found in those family members involved in linkage of actin to the cell surface. These domains are thought to be important for the regulation of junction assembly and specific attachment of intermediate filaments via associated adapter proteins. Recent developments have suggested the exciting possibility that these junctions, in addition to playing an important structural function in tissue integrity, are both acceptors and affectors of cell signaling pathways. Many desmosomal and hemidesmosomal constituents are phosphoproteins and in certain cases the function of specific phosphorylation sites in regulating protein-protein interactions is being uncovered. In addition, a more active role in transmitting signals that control morphogenesis during development and possibly even regulate cell growth and differentiation are being defined for cytoplasmic and membrane components of these junctions.—Green, K. J., Jones, J. C. R. Desmosomes and hemidesmosomes: structure and function of molecular components.** ***FASEB J.*** **10, 871–881 (1996)**

Key Words: cell junction · matrix connector · cytoskeleton

STRUCTURE AND DISTRIBUTION OF DESMOSOMES AND HEMIDESMOSOMES

The most prominent cell-surface attachment sites for intermediate filaments (IF)[2] in epithelial cells are desmosomes and hemidesmosomes, which mediate IF anchorage at sites of cell-cell and cell-substrate contact, respectively. By anchoring IF at sites of strong intercellular adhesion, desmosomes create a transcellular network throughout a tissue that is thought to resist forces of mechanical stress. This network in turn is attached to the basal aspect of the cell by molecularly distinct junctional structures called hemidesmosomes, which confer additional mechanical integrity to the tissue. Although providing mechanical integrity is thought to be a critical function of these junctions, it is clear that they are extremely dynamic structures that respond with exquisite sensitivity to environmental cues, allowing for tissue remodeling during development, differentiation, wound healing, and invasion. In addition to being modulated in response to their environment, cell junction molecules themselves play active roles in signal cascades initiated by extracellular matrix ligands and growth factors during development and in the adult.

As their names suggest, desmosomes and hemidesmosomes exhibit similar structural characteristics (Fig. 1). Each is composed of a tripartite electron-dense plaque structure specialized for IF anchorage. In the case of the desmosome, mirror image plaques sandwich a membrane core region, whereas a single plaque located at the basement membrane serves this function in the hemidesmosome (insets in Fig. 1). With one known exception, the molecules comprising these junctions are completely distinct, although certain components are evolutionarily related. Extracellularly, desmosomes are separated by a 30 nm space filled with material that represents in large part the extracellular domains of the single span transmembrane desmosomal cadherin molecules. Hemidesmosomes, on the other hand, are attached through an integrin-based mechanism to the underlying basement membrane and stroma.

Although desmosomes and hemidesmosomes are both found in epithelia where they associate with keratin-containing IF, desmosomes are thought to exhibit a more widespread tissue distribution. These intercellular junctions are also present in cardiac muscle where they an-

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[2]Abbreviations: IF, intermediate filaments; BP180, 180 kDa bullous pemphigoid antigen; GABEB, generalized atrophic benign epidermolysis bullosa: CP, cicatricial pemphigoid; JEB, junctional epidermolysis bullosa: IFAP, IF-associated protein: BP, bullous pemphigoid; APC, adenomatous polyposis coli.

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chor desmin-containing IF, and in the arachnoid and pia of meninges and follicular dendritic cells of the lymphoid system where they associate with vimentin-containing IF (1). In addition to skin and cornea, hemidesmosomes are also present in transitional epithelial cells (e.g., in bladder) and certain glandular epithelia (e.g., mammary gland epithelia and myoepithelial cells) (2, 3). The hemidesmosomes present in all these tissues are related not only by their ultrastructural character, but also by their composition. However, certain hemidesmosomal components also occur in simple epithelial cells such as those lining the gut that lack ultrastructurally defined hemidesmosomes (4–7). In these cases, it has been suggested that hemidesmosomal components are assembled into less organized multiprotein complexes for which some authors have now coined the term type II hemidesmosomes to distinguish them from the "classical" or type I hemidesmsome of basal epidermal cells (7).

Here we present the most recent developments addressing the molecular composition of these two junction types (shown schematically in **Fig. 2**), as well as the structure, function, and regulation of their constituents. This review will not be comprehensive, and we refer the reader to recent review articles for details of other components and a historical perspective of the subject (2, 3, 8).

The desmosome

The membrane molecules

Neighboring cells are thought to be adherent at desmosomes through interactions mediated by a relatively new division of the cadherin family of cell adhesion molecules known as the "desmosomal cadherins." This division includes the subclasses known as desmogleins and desmocollins (reviewed in refs 2, 9). Like the classic cadherins, desmosomal cadherins are single-pass, transmembrane-spanning glycoproteins with conserved regions of homology in the extracellular domain, thought to be involved in calcium binding and adhesion, and a major conserved region in the cytoplasmic domain required for binding to cytoplasmic adapter proteins. In the case of desmosomal cadherins, a protein called plakoglobin associates with this conserved region (reviewed in ref 10). The cytoplasmic domain of the desmogleins also harbors variable numbers of a 29 residue repeating motif of unknown function, unique to this cadherin subclass. Each desmocollin gene gives rise to two alternatively spliced

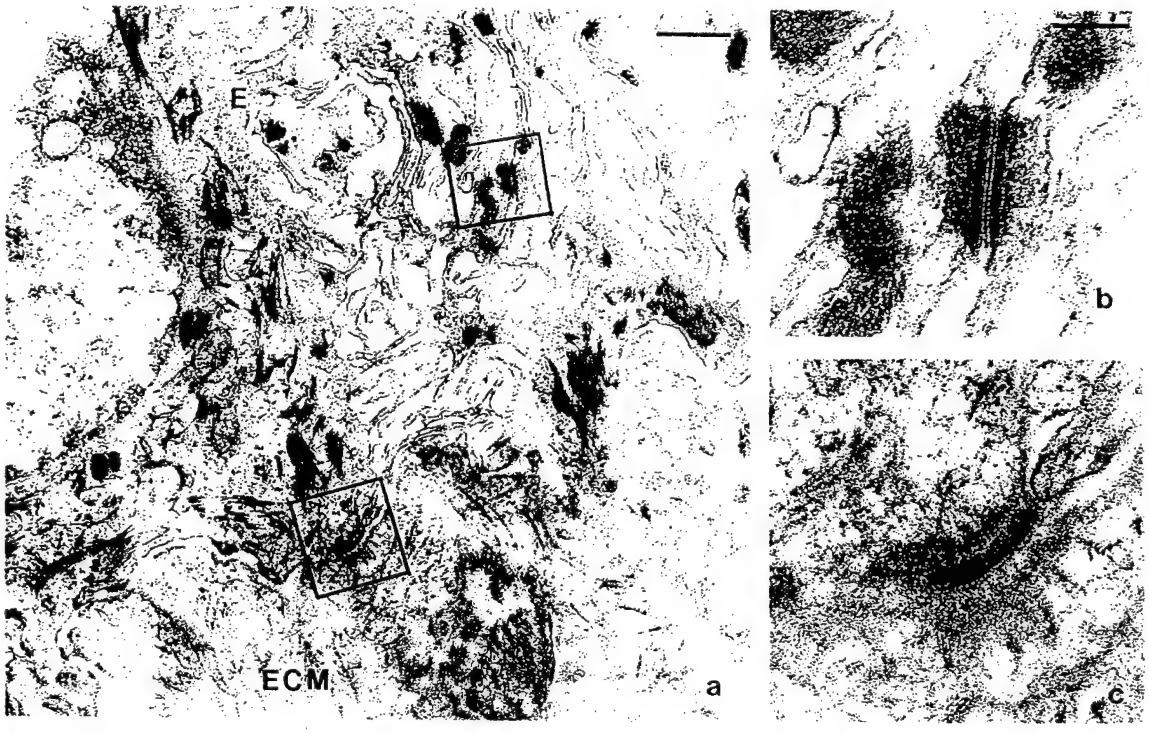

Figure 1. Electron micrographs of the basal layer of human epidermis (E, epidermal cell; ECM, extracellular matrix/dermis). *a)*; A region of interaction between two epidermal cells as well as epidermal cell-dermis association is shown. The upper box in panel *a* has been printed at higher magnification in panel *b*. Note the desmosome, with its characteristic electron-dense cytoplasmic plaques, lying either side of the contacting membranes of the epidermal cells. The lower boxed area in panel *a* is printed at higher magnification in panel *c* and shows a typical hemidesmosome. Like the desmosome in panel *b*, the hemidesmosome has a cytoplasmic plaque, but unlike the desmosome, each hemidesmosome abuts the dermis via the basement membrane. Panels *b* and *c* are at the same magnification. *a)* Bar, 500 nm; *b)* bar, 250 nm.

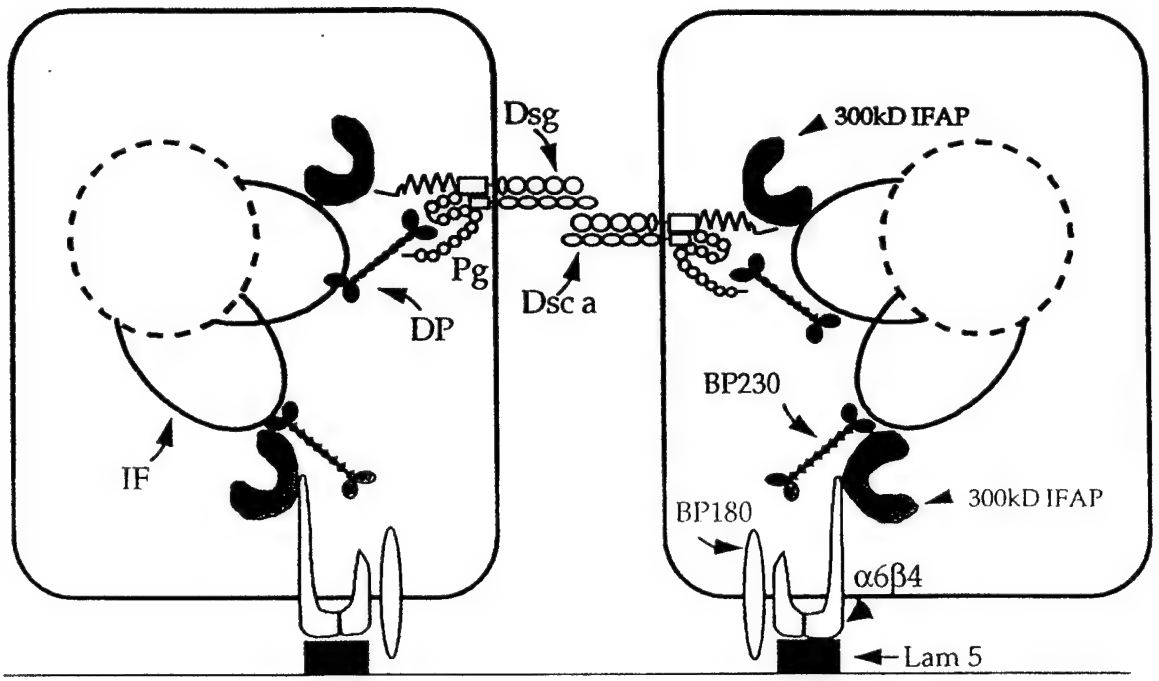

Figure 2. Schematic showing the major components of desmosomes and hemidesmosomes in two epithelial cells. One desmosome links the lateral domains of two epithelial cells while each is tethered to the underlying connective tissue via hemidesmosomes. Both the desmosome and hemidesmosome are connected to the intermediate filament (IF) cytoskeleton system which shows interaction with the surface of the nucleus (central shaded circle). We have taken some liberties in the diagram by indicating our ideas of how proteins may interact within these complex morphological entities. Dsg and Dsc a denote the cadherin-like desmogleins and desmocollins of the desmosome respectively. We only indicate the "long" or a isoform of desmocollin in the diagram. Pg, plakoglobin. desmoplakin, desmoplakin. Lam-5, laminin-5.

mRNA transcripts resulting in an "a" and "b" form, which differ only in the cytoplasmic domain, with the shorter "b" form containing 11 amino acids not in the "a" form. Although the functional significance of these two forms is unknown, the shorter desmocollin tail lacks the plakoglobin binding site present in the longer "a" form and in all desmogleins (11).

The recent identification of three desmocollin and three desmoglein genes has confirmed previous immunological evidence that the desmosomal glycoproteins are heterogeneous and expressed in tissue- and stratification-specific patterns (12–14). The desmosomal cadherins have been mapped to a small cluster on human chromosome 18q21; in the case of the desmogleins, they are tandemly linked in the order DSG1-DSG3-DSG2 from 5′ to 3′ mirroring their expression pattern from suprabasal to basal in stratified human epidermis (15).

Although the classic cadherins are typically thought to mediate calcium-dependent homophilic adhesion, the mechanism by which desmosomal cadherins function is largely unknown. Early work demonstrated that Fab′ antibody fragments against desmocollin inhibit desmosome assembly in MDBK cells (2). In addition, autoantibodies to desmoglein family members circulating in patients with the class of autoimmune epidermal blistering diseases called pemphigus have been demonstrated to be causative in mouse models of the disease, consistent with a role in disrupting intercellular adhesion (e.g., ref 16). Convincing experimental data supporting a role for individual desmosomal cadherins in calcium-dependent homophilic adhesion are lacking, however. A chimeric molecule with the Dsg3 extracellular domain fused to the E-cadherin cytoplasmic domain was shown to engage in weak homophilic adhesion that was not comparable to adhesion mediated by E-cadherin (17). Likewise, full-length desmoglein 1 and desmocollin 2, expressed with or without the associated plakoglobin molecule, are unable to support the level of adhesion mediated by E-cadherin (A. Kowalczyk and Green, unpublished results). The existence of multiple desmosomal cadherins within a single desmosome, as well as multiple tissue specific isoforms, suggests a functional complexity for these molecules not exhibited by their classic cadherin relatives. For instance, it is possible that the active form of desmosomal cadherin is a cell type-specific heterodimer that associates laterally within the junction. Such tissue-specific pairing may endow desmosomes with distinct adhesive and/or cytoskeletal linking roles.

Although the adhesive function of all possible desmosomal cadherin combinations has not yet been tested, the lack of demonstrable adhesion using traditional assays nevertheless highlights the importance of considering the role of other membrane molecules in desmosome function. Recently, a 22 kDa phospholipid-anchored molecule, called the E48 antigen, has been desmonstrated to confer adhesive properties on MOP8 cells (18).

The expression of E48 is restricted to specific tissues, so it is unlikely that this molecule plays a constitutive adhesive function in all tissues. However, the existence of as yet unidentified cell type-specific forms of E48 required for adhesion in other tissues should be considered.

The plaque molecules

The cytoplasmic plaque of desmosomes is complex and exhibits tissue-specific differences in both structure and composition. The constitutive components are plakoglobin and the most abundant component, desmoplakin. Although more minor, sometimes tissue-specific, components surely also play important roles in modulating adhesive or cytoskeletal interactions, emphasis will be placed on recent work dealing with the function and regulation of the major desmosomal components.

Plakoglobin

Plakoglobin belongs to an emerging gene family that also includes β-catenin, the cadherin-associated protein p120, and the tumor suppressor adenomatous polyposis coli (APC). Members of this family share at the core of their structure a series of repeating motifs first found in armadillo, a downstream effector in the wingless signal transduction pathway responsible for the establishment of segmentation polarity in *Drosophila* (19, 20).

As plakoglobin binds tightly to the cytoplasmic domains of both desmosomal cadherins, desmocollin (the larger "a" form) and desmogleins (11, 21–23), it may serve as a molecular link between the outer and inner portions of the desmosomal plaque. Consistent with this idea, deletion of the plakoglobin binding site in desmosomal cadherins abrogates the ability of these molecules to anchor IF at the plaque (11, 24).

Plakoglobin is not restricted to desmosomes but is a common component of adhesive junctions including microfilament associated cell-cell adherens junctions in epithelial and nonepithelial cells (1, 2, 10). This distribution likely reflects plakoblobin's ability to associate not only with the desmosomal cadherins, but also in separate complexes with the classic cadherins, albeit more weakly (25). A potential role for plakoglobin in adherens junctions is not clear. In fact, analysis of cross-linked junctional complexes from MDCK cells suggests that plakoglobin may not even be effectively recruited into Triton-insoluble adherens junctions in cells that have both desmosomes and adherens junctions (26).

In addition to their structural roles in intercellular junctions, members of the armadillo gene family act as signal transducers (19, 20). Armadillo is the most distal component of the signaling pathway mediated by a secreted protein in *Drosophila*, called wingless, which is homologous to the vertebrate Wnt growth factor family. Although, like its vertebrate relatives, armadillo binds to cadherins in cell-surface, adherens-type junctions, evidence suggests that a cytoplasmic rather than junction-associated form of armadillo proteins is active in signaling. Wingless results in the metabolic stabilization and accumulation of cytoplasmic armadillo, which is correlated with a change in its phosphorylation state due to the inactivation of the upstream serine/threonine zeste white kinase. Evidence that such a pool exerts a signaling effect in vertebrates comes from studies desmonstrating that overexpression by microinjection of β-catenin or plakoglobin mRNA into *Xenopus* embryos leads to the duplication of the embryonic axis, resulting in embryos with two heads, notochords, and neural tubes (27, 28). In the case of plakoglobin, this effect was abrogated by coexpression with the desmoglein cytoplasmic domain (28). The latter result suggests that the proper balance between cadherin-bound and unbound pools of plakoglobin/β-catenin is likely to be crucial for proper signaling during development. Along these lines, the extremely rapid degradation of noncadherin associated plakoglobin recently reported in fibroblasts ectopically expressing this protein may represent a general mechanism for controlling the accumulation of armadillo family members (22).

The mechanism by which the armadillo family members actually affect downstream changes in gene expression is unknown. Intriguingly, however, both β-catenin and plakoglobin have been shown to accumulate in the nucleus in overexpression experiments, and some have speculated that interaction of armadillo family members with a nuclear target may be involved in regulation of gene expression (27, 28). The tumor suppressor gene product APC, mutated in patients with the dominantly inherited disease familial adenomatous polyposis, also binds in a cytoplasmic complex with β-catenin or plakoglobin. The functional implications of this association for regulating cytoplasmic levels or signaling activity of these proteins are not known. However, this observation suggests that β-catenin and plakoglobin may be involved in regulating cell growth control in addition to development and differentiation (29, 30).

Plakophilin/band 6

What was previously called "band 6" in enriched preparations of desmosomes isolated from bovine tissues has now been identified as a plakoglobin-like molecule. Unlike plakoglobin, in vitro evidence indicates that this molecule may bind directly to IF polypeptides (31, 32). Band 6/plakophilin exhibits a broader tissue/cell distribution than previously recognized because it is found in the cytoplasm of several cultured lines, including those from simple epithelia. However, it is not a constitutive component of desmosomes and thus is unlikely to be absolutely required for IF anchorage. If plakophilin proves to be a signaling molecule like its armadillo family relatives, this could provide a potential mechanism for differentiation-specific signaling.

Desmoplakin

Although plakoglobin appears to play an important role in establishing contact with the IF cytoskeleton of des-

mosomes, more likely candidates exist for direct association with IF polypeptides. The most abundant and well-studied of these is desmoplakin. Desmoplakin is a large dumbbell-shaped molecule with a central α-helical coiled rod domain flanked by two globular end domains with distinct functions (33). So far, two alternatively spliced forms derived from a single desmoplakin gene have been reported. The smaller desmoplakin II product is more variably expressed, found at lower levels in nonstratified tissues and absent in certain tissues such as the heart.

Molecular mapping studies using transient transfection of constructs encoding specific domains of desmoplakin indicated for the first time that the carboxyl terminus of this molecule contains sequences that govern its association with IF networks (34). These initial observations were borne out by in vitro studies suggesting that this interaction is direct in the case of type II epidermal keratins, which interact with desmoplakin via amino-terminal sequences (35). Transient transfection studies have also mapped sequences required for association with the desmosomal plaque to the amino terminus of desmoplakin (36). Together, these studies suggest that desmoplakin is a functionally modular protein that acts as a molecular linker to anchor IFs at the desmosome; however, these domain mapping studies did not directly test this hypothesis. To address this idea directly, we recently used a dominant negative approach whereby a region of the amino terminus sufficient for localization and, presumably, binding to components of the desmosomal core was moderately overexpressed in stable A431 cell lines. The result was displacement of endogenous desmoplakin from the plaque and loss of IF anchorage, suggesting that desmoplakin is indeed required for this attachment (E. A. Bornslaeger and K. J. Green, unpublished observations).

Like plakoglobin, desmoplakin is a phosphoprotein. Recent evidence suggests that the interaction between the carboxyl terminus of desmoplakin and IF networks is regulated by phosphorylation of a serine residue located in a cAMP-dependent kinase consensus site 23 amino acids from the carboxy-terminal end of desmoplakin (37). This serine is within a region that had been shown to be required for interaction with keratin (but not vimentin) IF networks, and may represent a regulatory site for interaction with specific filament polypeptides (36). One possible function for such a phosphorylation event might be to prevent desmoplakin from becoming sequestered all along IF in the cytoplasm during its recruitment into desmosomes.

Other members of the desmoplakin gene family

BP230/plectin/IFAP300

Desmoplakin belongs to another emerging gene family whose members are involved in the organization or anchorage of IF networks. The first similarity identified was with BP230, a plaque component and candidate IF linker specifically found in hemidesmosomes that will be described in more detail (33). The third member is plectin, a known IF-associated protein (IFAP) with broad tissue distribution reported to be present in desmosomes and hemidesmosomes. Like desmoplakin, plectin's domain functions have been mapped using transient expression experiments, and the carboxy-terminal repeats have been demonstrated to associate with IF networks in cells (38). However, plectin has been shown to bind in vitro to many IF types, including nuclear lamin B as well as microtubule-associated proteins, α-spectrin and fodrin. Thus, this molecule may function as a universal linking protein.

Another potentially closely related protein called IFAP300 has also been demonstrated to be in desmosomes and hemidesmosomes (39). Similar to plectin, IFAP300 binds to cytoplasmic IF networks in fibroblasts in addition to being localized at both junction types in epithelial cells. Data supporting a central role for IFAP300 in IF anchorage will be discussed below. However, the specific roles each individual family member plays within a particular junction remain unknown. IFAP300 and desmoplakin are both IFAPs located in the desmosome, although desmoplakin is present at higher levels. One possibility is that IFAP300 augments interactions mediated by desmoplakin, contributing to the stability of IF interactions in desmosomes. The possible cell type specificity of family members may also be important for mediating interactions with different types of IF networks.

THE HEMIDESMOSOME

The membrane molecules

Hemidesmosome integrins

Integrins are heterodimeric matrix receptors each composed of an α and a β subunit. These receptors not only form part of the link that integrates the extracellular matrix and the cytoskeleton of cells, but also act to transduce signals (40). Until 1990, it was generally believed that cytoskeleton interactions of integrins were limited to the microfilament system of cells. However, in 1990/1991, several groups showed that the epithelial cell integrin α6β4 was concentrated in the hemidesmosome and therefore was spatially associated not with the actin cytoskeleton, but with keratin containing tonofilaments (reviewed in ref 3).

The α6 subunit

The α6 subunit can bind either the β1 or β4 subunit, but when given a choice it preferentially associates with β4 (41). In many epithelial cells, therefore, despite the presence of the β1 subunit, α6 is found exclusively associated with β4 integrin. There are several isoforms of the α6 integrin subunit (4, 6). Each isoform is synthesized as a 150 kDa polypeptide, which is then cleaved into "heavy" and "light" chains that associate via disulfide bonding. The two best-studied α6 isoforms, α6A and α6B, differ in their cytoplasmic domains, thus providing

a possible opportunity for interaction with differenct cytoplasmic components (6). The α6A isoform has been localized to tissues that possess typical hemidesmosomes (6); however, α6A is also found in gut epithelial cells lacking bona fide hemidesmosomes but that may assemble type II hemidesmosomes (6, 7). Likewise, α6B is found primarily in the kidney and certain epithelial glands where it may occur in a type II hemidesmosome-like structure (6, 7).

The β4 integrin subunit

The β4 integrin subunit is unique among the β integrins so far characterized because of the presence of an extended carboxy-terminal cytoplasmic tail of more than 1000 amino acids (4, 42). Alternative splicing of the β4 message gives rise to two different forms, each containing two type III fibronectin repeat motifs connected by the variable domain, which is also a site of proteolytic cleavage (4, 41). Most investigators in the field assume that the unusual structure of the β subunit cytoplasmic tail explains why α6β4 integrin is the only integrin heterodimer so far identified that is found associated with the IF cytoskeleton. This assumption is based on studies demonstrating that the cytoplasmic tails of subunits of other integrin heterodimers are often involved in anchorage of the actin cytoskeleton via one or more actin cytoskeleton-associated proteins. Indeed, there is now biochemical evidence to support this possibility, because an IF-associated protein IFAP300 that is a component of the hemidesmosome (39) binds β4 integrin in overlay assays (S. E. Baker and J. C. R. Jones, unpublished observations). Thus, IFAP300 may directly link IF to the β4 integrin cytoplasmic tail in much the same way that talin links actin filaments to the cytoplasmic domain of the β1 integrin subunit (40).

Antibodies directed against the external domains of the β4 integrin subunit inhibit hemidesmosome assembly and perturb the structural integrity of formed hemidesmosomes (43). The importance of the β4 integrin in hemidesmosome formation and stability has been confirmed by recent molecular genetic studies by Spinardi and co-workers (44, 45). These authors have made use of 804G cells, one of only a few cell lines that assemble hemidesmosomes in vitro (46). In their initial study they presented evidence that a region of 303 amino acids in the cytoplasmic domain of β4 is necessary for β4 subunit incorporation into hemidesmosomes, whereas the β4 extracellular domain is essential for β4 interaction with the α6 integrin subunit (44). These same workers have showed that overexpression of a tailless β4 integrin, lacking most of the cytoplasmic domain of the wild-type molecule, has a dominant negative effect that leads to perturbation of hemidesmosome organization (45). A mutation in the β4 integrin gene leading to premature termination of message transcription has now been discovered in one patient afflicted with the blistering skin disease junctional epidermolysis bullosa (47). If β4 integrin plays a role in nucleation of hemidesmosome assembly, its absence could explain a key histological feature of this disease, i.e., a decrease in the frequency of hemidesmosomes.

Recent data provide circumstantial support for the possibility that α6β4 integrin is involved in signal transduction. β4 Integrin is physically associated with one or more protein kinases; upon interaction of the α6β4 with its extracellular ligand, β4 becomes phosphorylated on tyrosine (48). Furthermore, a tyrosine phosphorylation site in the cytoplasmic domain of β4 has been shown to be required for its association with other hemidesmosome components (48). This site lies in a tyrosine-based activation motif or TAM consisting of two possible phosphorylatable tyrosine residues, followed by a leucine at position +3 (48). In addition, a separate tyrosine phosphorylation event in β4 appears to trigger binding of the signaling adaptor molecule Shc, which upon phosphorylation recruits the adaptor Grb2 (48). This study suggests that hemidesmosome integrins may mediate signaling events from the matrix to an epithelial cell in a manner similar to other integrin receptors such as the fibronectin receptor α5β1 (reviewed in ref 40).

The 180 kDa bullous pemphigoid antigen (BP180)

Autoantibodies circulating in some patients afflicted with bullous pemphigoid (BP) recognize a 180 kDa hemidesmosomal protein, variously termed BP180 or BPAG2 (bullous pemphigoid antigen 2). The cytoplasmic domain of this type II membrane protein (i.e., its amino terminus is located in the cytoplasm) is separated by a membrane domain from a short extracellular stretch of highly charged amino acids leading to a region containing a series of GLY-X-Y or collagen-like repeats (49, 50). Based on its collagen-like structure, BP180 has been referred to by some investigators as type XVII collagen. It is generally assumed that the collagen extracellular domain is involved in interactions between BP180 and components of the basement membrane, although the nature of such interactions is yet to be defined.

The importance of BP180 for epidermal-connective tissue interactions has been highlighted by the identification of inherited and autoimmune skin diseases that target the BP180 gene or protein. BP180 is missing from the skin of individuals suffering generalized atrophic benign epidermolysis bullosa (GABEB) (51,52). In one case this has been shown to occur because a mutation in the BP180 gene leads to premature transcription termination of the BP180 message, and thus to a lack of BP180 protein in the skin (52). At the electron microscopic level, hemidesmosomes are either missing or present in a rudimentary state in the skin of GABEB patients; presumably this weakens the attachment of epidermal cells to the basement membrane and leads to blistering. BP180 is also a target for pathogenic antibodies in two autoimmune diseases, bullous pemphigoid and herpes gestationis (53). In particular, the perimembrane noncollagenous extracel-

lular domain of BP180 contains an epitope recognized by some (but not all) BP180 autoantibodies (53). Giudice and co-workers (54) have shown that neonatal mice injected with antibodies against this same epitope develop lesions histologically identical to those seen in bullous pemphigoid patients.

A region of 36 amino acids at the amino terminus of BP180 is required for its polarization in the plasma membrane (55). On the other hand, the perimembrane 27 amino acid noncollagenous domain of BP180, the target for pathogenic autoantibodies, appears to be essential for interactions between BP180 and other hemidesmosomal components. With what hemidesmosome element (or elements) does BP180 interact? Using a molecular genetic approach, Hopkinson et al. (55) have provided evidence that BP180 may interact with α6 integrin because BP180 associates morphologically with the α6 integrin subunit regardless of its β partner and α6 antibodies coprecipitate BP180. Indeed, these same workers have speculated that pathogenesis of BP involves autoantibody induced disruption of BP180-α6 integrin interaction leading to perturbation of the structural integrity of the hemidesmosomes. This would parallel the disruption of the hemidesmosomes observed in in tissue explants treated with a function blocking α6 integrin antibody (43).

Matrix molecules

Laminin-5, also referred to as GB3 antigen, epiligrin, and kalinin, is a newly characterized member of a growing family of laminin heterotrimers and is composed of three subunits termed α3, β2, and γ2 (56). Immunoelectron microscopy has revealed that laminin-5 is concentrated in the basement membrane zone immediately underlying each hemidesmosome in stratified squamous epithelial tissues (3). However, note that some if not all of the chains of laminin-5 are expressed by lung epithelial cells that do not possess bona fide hemidesmosomes but that may assemble type II hemidesmosomes (5, 7).

Laminin-5, like other matrix proteins, is promiscuous with regard to its cell receptors (57). For example, in skin cells maintained in vitro, α3β1 appears to initiate cell binding to laminin-5 (58). α6β1 may also act as a laminin-5 receptor in some tissue cultured cells whereas α6β4 integrin is involved in establishment of so-called long term stable anchoring contacts or hemidesmosomes on laminin-5 rich matrices (57–59). The physiological relevance of these laminin-5/integrin interactions is not yet clear because they may not all occur in vivo. However, it is possible that such promiscuity reflects a functional diversity in laminin-5 that in some way is modulated by the nature of its cell-surface associations. This may help explain why a protein involved in formation of stable anchoring contacts, is also found at sites of active cell motility such as the invasion front of colon carcinomas (60). In this regard, the apparent receptor promiscuity of laminin-5 may result from proteolytic processing of its component chains and thus presentation of previously masked receptor binding sites (61). Alternatively, there is now evidence for the existence of laminin-5 chain isoforms that may exhibit distinct receptor specifities (62).

Laminin-5, like BP180, is the target molecule for antibodies in an autoimmune disease (cicatricial pemphigoid, CP) and has been shown to be deficient in the skin of patients with a genetic disease termed junctional epidermolysis bullosa (JEB) (63–67). CP and JEB are characterized by loss of cohesion between an epidermal cell and the basement membrane as well as the absence of hemidesmosomes, suggesting a role for laminin-5 in hemidesmosome assembly. Consistent with this idea, human SCC12 (squamous cell carcinoma) keratinocytes maintained under normal culture conditions fail to form hemidesmosomes. In contrast, SCC12 cells are induced to assemble hemidesmosomes when maintained on a laminin-5 rich matrix secreted by 804G cells (59). Matrix-induced assembly of hemidesmosomes in SCC12 cells involves reorganization of hemidesmosome components, including α6β4 integrin and BP180, along the cell-matrix interface, resulting in a distribution pattern that overlies the laminin-5 components in 804G cell matrix. In other words, 804G matrix contains a structural cue which when transduced to overlying cells, most likely via α6β4 integrin, can trigger hemidesmosome assembly. It will be interesting to address whether the mechanism of hemidesmosome assembly in this system involves a similar signaling pathway involving phosphorylation of β4 integrin as described by Mainiero et al. (48).

The plaque molecules

BP230 and BP230 isoforms

Autoantibodies directed against a 230 kDa plaque component of the hemidesmosome are found in the majority of bullous pemphigoid serum samples (68). Because it was characterized before BP180, it is sometimes called BPAG1, although we shall refer to it as BP230. BP230 localizes within the region of the hemidesmosome plaque to which keratin IF attach (3), providing a first clue that BP230 may be involved in IF cell-surface anchorage. This idea is further supported by the discovery that BP230 belongs to a family of proteins that include desmoplakin and another IF-associated protein called plectin, all possessing striking sequence similarities in their carboxyl terminus, a likely site of binding to IF (33). Indeed, isolated BP230 molecules even bear a superficial resemblance to desmoplakin because both possess a central rod domain with globular ends (69).

Experimental evidence that BP230 plays a role in organizing the IF cytoskeleton comes from studies of mice in which BP230 has been ablated by targeted homologous recombination (70). Hemidesmosomes in the epidermal cells of these mice lack the innermost cytoplasmic region of the hemidesmosomal plaque and exhibit few, if any, associated IFs (70). This is not entirely surprising given the localization of BP230 antigen and speculation that

this protein binds IF in a manner similar to desmoplakin and plectin. However, an unexpected feature of the BP230 knockout mice is that they develop a neuropathy with similar pathological and behavioral features to mice homozygous for the *dystonia musculorum (dt/dt)* mutation. This is not just a coincidence, because the dt allele has been mapped to the same chromosomal location as the gene encoding the BP230 protein (71). Furthermore, sequence analysis of a candidate dt gene ("dystonin") has revealed that it encodes a BP230 isoform ("dystonin") differing in its amino terminus from the BP230 protein expressed in epidermal cells (71). The putative amino-terminal domain of this neural isoform shows sequence similarity to several actin binding proteins including β-spectrin and α-actinin, suggesting that the protein product of the "dystonin" gene may bind both actin-containing microfilaments and IF (71). The possibility that "dystonin" is found in the nervous system equivalent of the hemidesmosome is an intriguing but untested idea.

"Dystonin" is likely to be just one of a family of BP230 isoforms. Hopkinson and Jones (72) have characterized a 280 kDa isoform ("BP280") in a pancreatic carcinoma cell line that is expressed in a variety of cultured epithelial cells and epidermis but apparently is absent from cells of mesenchymal origin (72). Although BP280 lacks the carboxy-terminal IF binding domain, discussed above, it distributes with the filamentous cytoskeleton in certain epithelial cells (72). This observation suggests that more than one domain may be involved in the interaction between the cytoskeleton and BP230 and its isoforms. Such a possibility will need to be tested experimentally, for example, using the type of molecular genetic approach that has proved so successful for identifying functional domains in desmoplakin, plectin, and BP180 (34, 38, 55).

IFAP300/HD1/plectin

As already discussed, BP230 is a strong candidate for an IF-hemidesmosome plaque linker. However, the IF-associated protein IFAP300 has also been identified in the hemidesmosome, in addition to the desmosome (39). Because this protein binds IF in vitro, it could also function to link IF to the cell surface. By analogy to the focal contact, which contains several actin binding proteins, BP230 and IFAP300 could well play complementary roles in IF-hemidesmosome interactions. Indeed, IFAP300 and BP230 may be more than simply functionally related because a partial sequence of IFAP300 indicates that it possesses some sequence similarities to plectin, a member of the same family as BP230 and desmoplakin (39).

IFAP300 was first characterized as a 300 kDa protein associated with the vimentin cytoskeleton of fibroblast cells (73). It is immunologically related, if not identical, to a high molecular weight protein of bovine corneal hemidesmosomes termed HD1, which has also been proposed as an IF-hemidesmosome linker (74; J. C. R. Jones and K. Owaribe, unpublished observations). HD1, like IFAP300, is not restricted to hemidesmosomes but is expressed in numerous epithelial cell types as well as neuronal cells (74). Because HD1 is often coexpressed with α6β4, it has been proposed that HD1/α6β4 complexes define a type II hemidesmosome, as we have already mentioned (7).

ISSUES RISING AND PERSPECTIVES

Significant advances have been made in the identification and characterization of desmosome and hemidesmosome components. In many cases, molecular genetic analysis has allowed functional assignments for individual protein domains. It is likely that state of the art assays such as the yeast two hybrid approach will continue to identify protein-protein interactions in these junctions as well as new components that may be present at substoichiometric levels. In addition, high-resolution structural studies such as that recently reported for the N-cadherin extracellular domain also promise to reveal new information regarding the structural basis of cell adhesion, and ultimately of cytoplasmic interactions (75).

A major challenge will be to define signaling pathways that regulate junction assembly and dissolution. Both desmosome and hemidesmosome assembly involve a spatially and temporally regulated succession of protein-protein interactions. It is generally believed that assembly of junctional plaques is triggered by the lateral association or clustering of transmembrane protein complexes. In the case of the desmosome, this idea is supported by the studies of Troyanovsky (76) demonstrating that clustering of connexin-desmosomal cadherin tail chimeras in the plane of the membrane recruits other plaque components and leads to anchorage of the intermediate filament cytoskeleton. Clustering of hemidesmosomal integrins is also likely to play a role in nucleation of junction assembly as well as recruitment of cytoplasmic components, including IF bundles (45, 59).

What actually leads to clustering of desmosomal cadherins during normal assembly is not well understood. In cultured cell systems, cell contact and calcium lead to a temporal sequence that begins with homophilic adhesion via classic cadherins and proceeds with adherens junction assembly. Desmosome assembly begins shortly thereafter, and although data from different systems sometimes conflict, it appears to depend on a balance in protein kinase and phosphatase activity (see, for example, ref 77). Data from calcium induction experiments contrast with recent evidence demonstrating that half desmosomes can be assembled on their own without a counterpart on an adjacent cell and suggesting that cell contact/calcium serves not as a signal, but simply allows adjacent half desmosomes to interact through the desmosomal cadherin's extracellular domains (78).

Regulating junction dissolution may be equally important, particularly in processes such as wound healing and

invasion. A correlation has been made between growth factor-dependent tyrosine phosphorylation of plakoglobin and a more invasive cell state (79); plakoglobin has also been shown to bind to tyrosine kinase growth factor receptors (80). Thus, in tumors where adhesion is compromised and motility is enhanced, phosphorylation of the catenins or plakoglobin may contribute to loss of cadherin function.

The function of individual proteins, though increasingly well defined in cultured cells, for the most part still needs to be determined at a tissue level. In the case of hemidesmosomes, recently identified inherited diseases are providing important functional information regarding protein components of hemidesmosomes. Although autoimmune diseases that target the desmoglein family of desmosomal molecules are well recognized, so far no genodermatoses have been attributed to mutations in desmosomal components. However, certain genodermatoses have been narrowed down to the desmosomal cadherin chromosome, and within the next 5 years this frontier is sure to be broken (81). In addition, studies in a variety of developmental model systems are beginning to elucidate the function of individual proteins in morphogenesis and differentiation in complex systems and tissues in vivo, and in certain instances are yielding unpredicted and surprising results. For example, gene ablation studies in mice have led to the discovery that a BP230 isoform has unexpected functions in the nervous system (70), whereas studies in flies and frogs have revealed that plakoglobin and its relatives play a central signaling role in embryogenesis (20). Advances in in vitro and cell culture techniques, along with the insights provided by more complex model systems, promise to make the near future a time of great strides in our understanding of cell junctions. FJ

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Expression of Hemidesmosomes and Component Proteins Is Lost by Invasive Breast Cancer Cells

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Hemidesmosomes are multiprotein structures that attach basal cells of stratified epithelia to basement membranes. Although normal human breast epithelia are not stratified, we observed expression of electron-dense hemidesmosomes and hemidesmosome protein components by breast epithelial and myoepithelial cells at the basal lamina* in vivo. *Primary cultured normal human breast epithelial cells also contained hemidesmosomes and component proteins, and could be used as a model for hemidesmosome assembly and regulation. In these cultured cells, hemidesmosome proteins were expressed and localized basally in an unvaried temporal pattern, and electron-dense hemidesmosomes were not seen until the final protein was localized to the cell base. In addition, rate of localization was influenced by confluence, doubling time, and extracellular matrix. Invasive breast cancer cells did not express hemidesmosomes or most of the component proteins* in vivo. *In carcinoma* in situ, *cells away from the basement membrane lacked hemidesmosomes and hemidesmosome proteins, and cells at the basement membrane exhibited abnormalities of hemidesmosome protein expression. Primary human malignant breast cells in culture exhibited a mix of hemidesmosome phenotypes. These data suggest that hemidesmosomes may be important subcellular structures in determining the cytoarchitecture of the breast epithelium. Further, their downregulation may influence cytoarchitecture remodeling closely linked with cell cycle, motility, and extracellular matrix interactions; and their loss in carcinoma may be associated with loss of normal cytoarchitecture. (Am J Pathol 1995, 147:1823–1839)

A body of literature has been accumulating suggesting that adhesion molecules, those proteins and other substances that cells use to adhere to their substrate, may play a role in cancer cell invasion and metastasis.[1, 2] Data have suggested on the one hand that cancer cells may exhibit reduced adhesion molecule expression or function resulting in release of their substrate, and freeing cells to pile up or migrate. On the other hand, carcinoma cells may acquire expression of new adhesion molecules to grip the basement membrane (BM) in order to traverse it, or to adhere to tissues at sites of metastasis.[3, 4]

We and others have previously addressed this subject in the breast in studies of the integrin class of cell adhesion molecules, several of which are present in normal breast tissue, but reduced or absent in carcinoma (see, e.g., refs. 5–9). Hemidesmosomes (HD) are another adhesion structure to study in this context. They are found only in epithelial cells,[10] which are usually stationary, but not in cells such as fibroblasts or macrophages, which wander (for recent reviews see refs. 11–13). Further, HD, more than other adhesion structures, may mediate firm, relatively immobile attachment to the BM,[14, 15] preventing the cell movement characteristic of invading malignant cells.

HD loss has been seen in cutaneous basal cell carcinoma.[16, 17] In addition, the $\alpha 6\beta 4$ integrin is a

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component of HD[18–20], and we and others[5–7, 21, 22] found that the $\alpha 6$ and $\beta 4$ integrin subunits were not expressed in some malignant mammary epithelial cells.

Although HD have been most commonly described in stratified epithelia, and the breast parenchyma is not normally stratified, ductal epithelia from various sources have been shown to contain HD.[13] It is thought that epithelia that contain HD may be those under greater shear stress. The breast contains a ductal epithelium that undergoes great shear stress during lactation, and some electron microscope (EM) studies have suggested that at least breast myoepithelial cells, and perhaps luminal cells that contact the BM, may have HD.[23–28] It is not clear, however, whether these electron-dense structures contain the same molecular components described in other well studied HD.

For this paper we studied normal and malignant breast cells in tissue sections and in culture for the presence of HD and some of their constituent and associated proteins including the M_r 180 and 230 bullous pemphigoid antigens, an M_r 200 protein, and collagen VII. Normal human mammary epithelial cells (HMEC) had HD *in vivo* and in culture, and expressed the expected spectrum of HD proteins, whereas invasive carcinoma cells lacked HD *in vivo*, and malignant cells in culture exhibited defects in HD assembly. In addition we used normal HMEC in culture as a model to study HD assembly and regulation.

Materials and Methods

Tissues

Breast tissues were obtained from Northwestern Memorial Hospital, Evanston Hospital, or the Cooperative Human Tissue Network of the National Cancer Institute. All malignant tissues used were infiltrating ductal carcinoma varying from grades I to III, and were from patients with from 0 positive lymph nodes to 10/10 positive level III nodes. Normal breast tissue was derived from such cancer patients, and from reduction mammoplasties. In addition, one sample of normal lactating tissue was obtained as the "normal" tissue from one of the mastectomies. Specimens were obtained fresh from surgery, and processed for EM, frozen sections, or cell culture.

Cell Culture

Mammary epithelial cells were derived from 11 infiltrating ductal carcinomas, 7 reduction mammoplasties, and sites distant from 2 of the carcinomas. Epithelial or carcinoma cells were culled and grown on plastic by the method of Stampfer[29] with revisions for tumor cell growth[30] in MCDB-170 medium (American Bioorganics, Inc., Niagara Falls, NY) plus serum-free supplements.[29]

Mammary epithelial cell strains were determined to be epithelial by their expression of desmosome proteins by immunofluorescence (IF) and determined to be malignant by their ability to proliferate in the absence of certain growth factors, in the presence of transforming growth factor-β, and at high cell concentrations, and their inability to form three-dimensional structures on Matrigel BM-like substance.[30]

804G cells (a rat urinary bladder carcinoma cell line) were maintained in culture as previously described.[31] These cells were utilized for their capacity to produce laminin-5-rich extracellular matrix (ECM), as described below.

Immunofluorescence

Tissues fresh from surgery were snap-frozen in liquid nitrogen and stored at −70°C until use. Pieces of frozen tissue were embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN), sectioned on a Tissue-Tek cryostat (Miles Laboratories) to a depth of ≈8 μm, and placed on poly-L-lysine (Sigma Chemical Co., St. Louis, MO)-coated glass microscope slides (VWR, Media, PA). Sections were fixed for 5 minutes in −20°C acetone (Mallinckrodt, Paris, KY) and air-dried. Tissues from seven infiltrating ductal carcinomas, seven normal specimens, and one sample of normal lactating tissue from a cancerous breast were used.

Cultured cells were grown on glass coverslips (VWR) in six-well plates (Falcon, Lincoln Park, NJ) at 10^4 to 5×10^4 cells per well. After three washes in phosphate-buffered saline (PBS) pH 7.4 containing 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, 1.15 g/L Na_2HPO_4, cells on coverslips were fixed for 3 minutes in −20°C methanol (Fisher Scientific, Fair Lawn, NJ) and air-dried. Cells from six carcinomas and four normal tissues (three reduction mammoplasties and one mastectomy) were used.

IF was performed as previously described.[5] Slides were observed and photographed using a Leitz Laborlux D fluorescence microscope and TMAX 100 film (Eastman Kodak Co., Rochester, NY). All photographic exposures were for 1.5 minutes.

The following primary antibodies were used: serum from a bullous pemphigoid patient containing human autoantibodies reactive primarily with an M_r

230 plaque protein of the HD,[32] 180 mouse monoclonal[33] and J17 rabbit polyclonal[34] antibodies to an M_r 180 transmembrane HD protein, 6A5 mouse monoclonal antibody to an M_r 200 HD protein,[35] 9C3 mouse monoclonal antibody, and EBA human autoantibody[36] to collagen VII anchoring fibril protein (which is also the epidermolysis bullosa aquisita antigen). Secondary antibodies included fluorescein-conjugated anti-mouse IgG + IgM, anti-human IgG + IgM and anti-human IgM; and rhodamine-conjugated anti-mouse IgG + IgM and anti-rabbit IgG (Kierkegaard and Perry, Gaithersburg, MD). Antibody concentrations were determined on the basis of concentration curves.

EM

EM was performed using standard methodology.[37] Briefly, 1 mm^3 tissues fresh from surgery were fixed in 2.5% glutaraldehyde in 0.1 mol/L Sorensens's phosphate buffer, postfixed in 1% OsO_4, stained in 2.5% uranyl acetate, dehydrated and infiltrated with propylene oxide, and embedded in Spurr epoxy resin (all from Electron Microscopy Sciences, Fort Washington, PA). Thick sections were examined by a pathologist to confirm diagnoses and find regions of carcinoma. A total of 12 carcinomas and 14 normal tissues (2 from reduction mammoplasties and 12 from cancer patients) were used for EM.

Cultured cells were grown on glass coverslips or on Matrigel (Collaborative Research, Bedford, MA)-covered glass coverslips in six-well plates at 10^4 to 5 $\times$ 10^4 cells/well, processed similarly to tissue samples and embedded in Epon-araldite resin 812 (Tousimis, Rockville, MD, or Fisher Scientific). Samples from five malignant cell strains and five normal cell strains (cells from four reduction mammoplasty patients, and normal cells from one cancer patient) were processed.

Thin sections were cut on a Reichert Ultracut E microtome (Reichert Instruments, Buffalo, NY), mounted on copper grids, stained with uranyl acetate and lead citrate (Electron Microscopy Sciences) and viewed at 80 kV in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA).

Growth of Cells on Matrigel BM-Like Substance

35 mm^2 wells were coated with 500 μl undiluted cold Matrigel, then placed at 37°C for at least 30 minutes. Matrigel, a substance produced *in vivo* by the Engelbreth-Holm-Swarm murine tumor, resembles the lamina lucida portion of the BM, and contains (among many substances) mainly laminin, collagen IV, and proteoglycans.

Preparation of Laminin-5-Rich Matrices from 804G Cells

804G matrix was prepared as described previously.[31] Briefly, 804G cells were grown to confluence on glass coverslips, the culture medium was aspirated, and the cells were washed in sterile PBS and removed from their matrix with 20 mmol/L NH_4OH for 5 minutes followed by three PBS washes. HMEC were plated on the matrix in their usual medium. After 24 hours, HMEC on 804G matrix were fixed in 2.5% glutaraldehyde and processed for EM as above.

Results

In Vivo *(Tissues)*

Mammary epithelial cells *in vivo* contained electron dense HD at their basal plasma membranes where they were in contact with the BM (Figure 1). These triangular-shaped plaques were associated with intermediate filaments intracellularly, and with anchoring filaments and anchoring fibrils extracellularly. HD were seen in all cells where contact with the BM could be shown. This included luminal cells that reached from the lumen to the BM (Figure 1, D and E) and myoepithelial cells that did not appear to reach the lumen (Figure 1B).

In all intraductal regions of malignant tumors, cells in contact with the BM also exhibited apparently normal HD (Figure 2). Some of these cells may have been normal myoepithelial cells, but many had morphological features of malignant cells and lacked the contractile filaments, large numbers of mitochondria, and shape typical of myoepithelial cells. All cells in contact with the BM had HD, whereas cells not at the BM contained no HD, although numerous desmosomes and some adherens junctions were seen (Figure 2).

In all invasive regions examined, no HD were seen in any cells (Figure 3) whether single cells, in small groups, or invading en masse and attached to one another with desmosomes.

Because HD have not been well studied in the breast, we looked by IF microscopy at expression of several of the protein components of HD previously described in skin. The anchoring fibril protein collagen VII (Figure 4C), M_r 180 (Figure 5A), and M_r 230 bullous pemphigoid antigens, M_r 200 protein (Figure 6C), and the α6 and β4 integrin subunits[5] were all

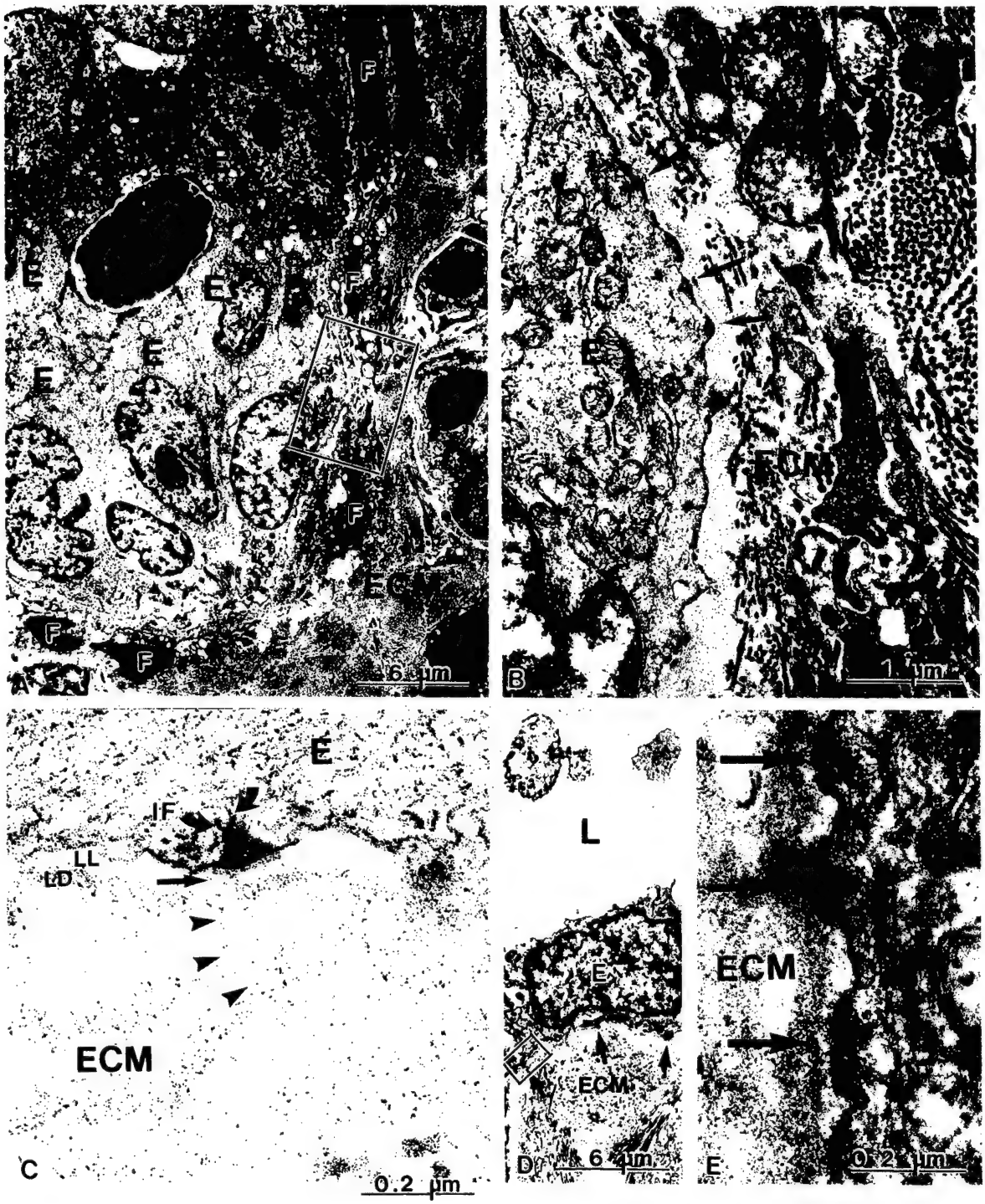

Figure 1. *The presence of hemidesmosomes in normal breast epithelium by electron microscopy.* (A) *A normal duct in cross section.* (B) *Higher magnification of box in* A *shows HD* (arrows) *at the bases of ductal epithelial cells.* (C) *Higher magnification of an HD showing intermediate filaments* (*IF,* curved arrows), *anchoring filaments* (arrow) *and anchoring fibrils* (arrowheads *along the length*). *LL, lamina lucida; LD, lamina densa.* (D) *A luminal cell reaching from lumen to basement membrane containing basal HD* (arrows). (E) *Higher magnification of the box in* D *showing HD* (arrows). *L, lumen;* E, *epithelial cell; F, fibroblast.*

present in normal ducts at the basal aspects of cells (β4 and the M_r 200 protein exhibited a basolateral distribution).

We next wished to determine whether abnormalities of HD protein expression would be seen in breast carcinoma. Two patterns emerged. The first

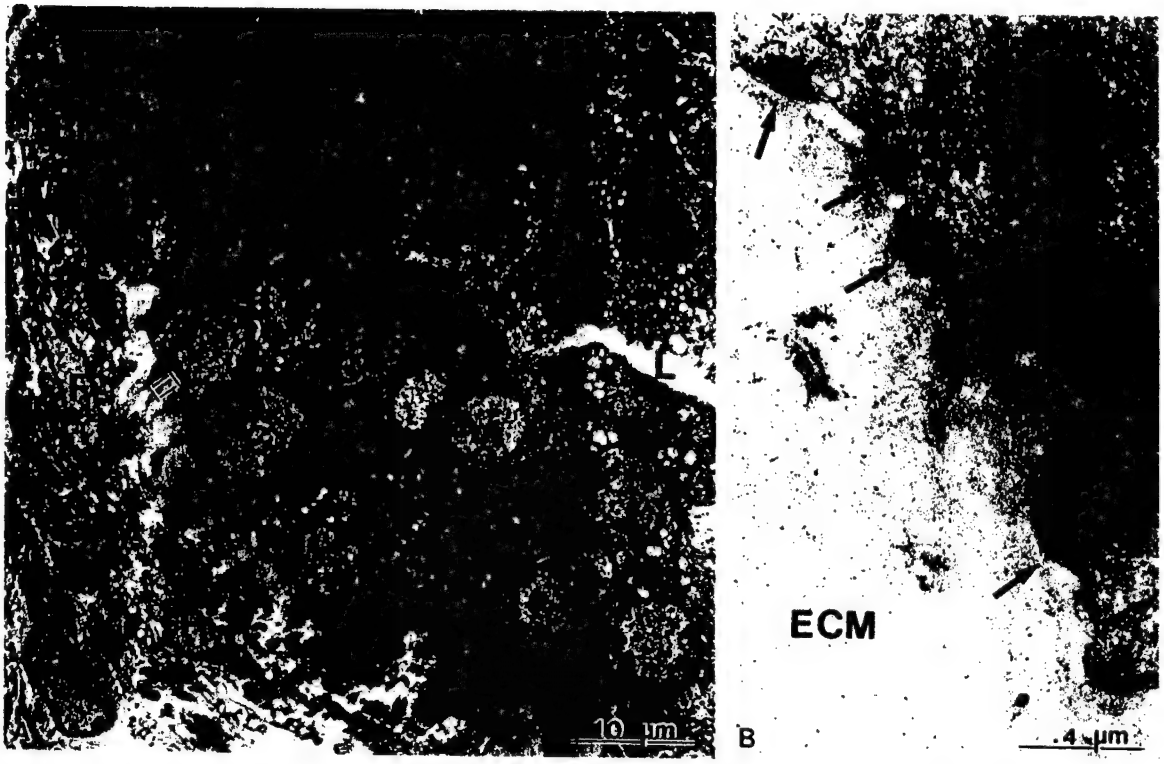

Figure 2. *Presence of HD in breast carcinoma* in situ *by EM.* (A) *An intraductal carcinoma showing many layers of cells within a duct with very little lumen (L) but surrounded by a BM.* (B) *Higher magnification of the box in* A *shows multiple HD* (arrows). *C, carcinoma cell; F, fibroblast.*

pattern, seen with collagen VII. the M_r 180 and 230 proteins and the α6 integrin subunit[5] is illustrated for collagen VII in Figure 4. Staining for the HD proteins was seen at the BM in normal ducts and in intraductal carcinoma. Cells piled up within cancerous ducts, and invasive cells exhibited no staining. A slight variation of this pattern was seen with the M_r 180 protein from which staining in all but one patient exhibited the aforementioned pattern, whereas the malignant cells from one patient exhibited no staining for the M_r 180 protein, even in regions of intraductal carcinoma (Figure 5, C and E).

The second pattern, seen with the M_r 200 protein and the β4 integrin subunit,[5] is illustrated in Figure 6. Staining was seen at the BM in normal ducts. In both carcinoma *in situ* and invasive carcinoma, regions of staining were seen and regions devoid of staining were also observed. For any one patient there were regions of intraductal carcinoma outlined by staining, and intraductal carcinoma without staining, and invasive cells that stained or did not stain.

Cultured Cells

Because we saw abnormalities of HD and HD protein expression *in vivo*, we decided to study cultured HMEC as a prelude to use of these cells for experimental manipulations.

Normal HMEC in culture exhibited electron-dense, apparently normal, HD by 2 weeks in culture (Figure 7). Malignant cell strains varied in their phenotype. For three patients, no cells could be found with HD; for one patient all cells had abundant, apparently normal HD; and the cells of one patient were a mix of cells with no HD, abundant HD, and a few HD.

We next stained cultured HMEC to see if the electron-dense HD seen by EM might also contain the expected protein components. Normal HMEC expressed the anchoring fibril protein collagen VII in a basal secreted pattern (Figure 8), and the M_r 200, 230, and 180 proteins in rows of basal tick-mark-shaped plaques (Figures 9 and 10). The α6 and β4 integrins are also expressed in this manner and colocalize with one another.[5]

Malignant HMEC also expressed all of the HD-associated proteins tested for. However, several abnormalities of expression were consistently noted. When malignant cells were plated on coverslips, it took 2 to 3 weeks to see a basal secreted pattern of collagen VII staining. whereas normal cells showed this pattern in less than a week. In time course experiments (Figure 8) we found that within 1 day normal HMEC produced

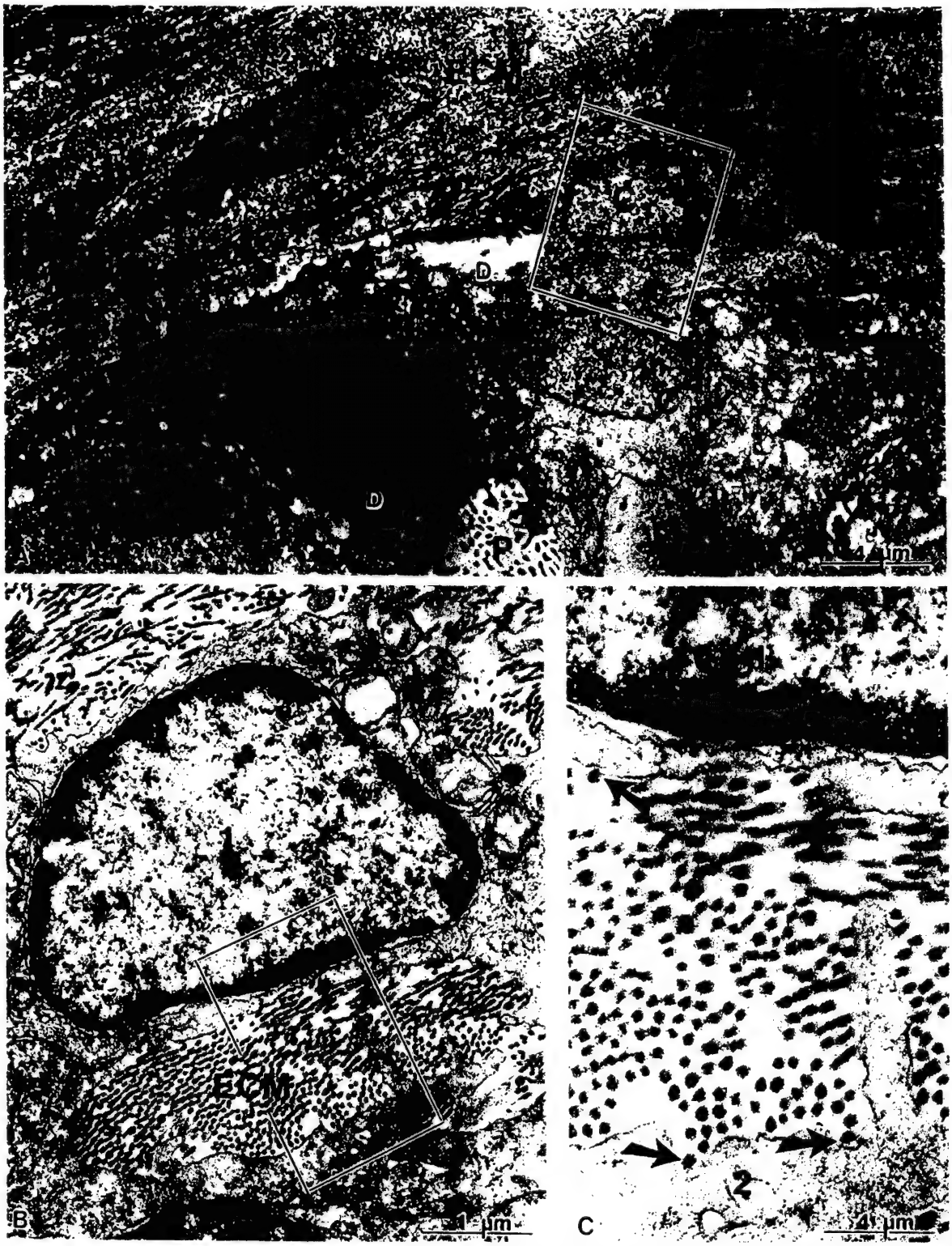

Figure 3. *Absence of HD in invasive breast carcinoma.* (A) *A group of invasive cells is pictured. Note the absence of a BM at the epithelial: ECM border* (curved arrows). *presence of desmosomes* (D. straight arrows). *A cell, apparently at the invasive front, is surrounded by ECM on three sides* (box). *C, carcinoma cell; F, fibroblast; P, pseudolumen.* (B) *Higher magnification of the box in* A *shows two invasive cells (1 and 2) abutting the ECM without any HD.* (C) *Higher magnification of box in* B *shows cells (1 and 2) with cell membranes in direct contact with collagen* (arrows) *without any intervening BM or HD.*

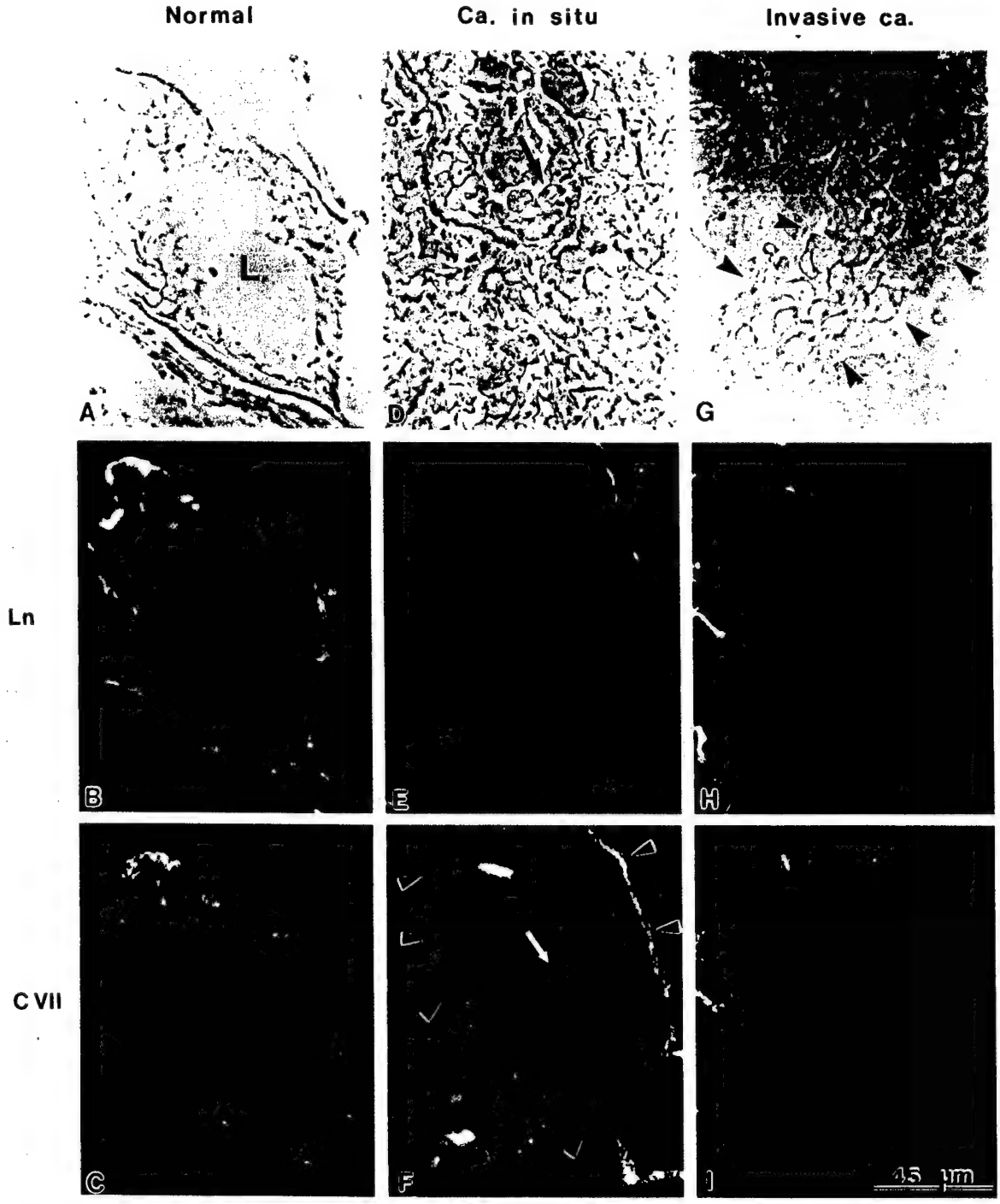

Figure 4. *Expression of the anchoring fibril protein, collagen VII in normal duct and carcinoma* in situ, *but not invasive carcinoma by immunofluorescence.* (A–C) *A normal duct in cross-section* (A) *has a BM as determined by laminin staining* (B) *and expresses basal collagen VII* (C). (D–F) *An intraductal carcinoma* (D) *has a BM around the entire group of cells* (E) *and exhibits collagen VII staining* (F) *only at the basement membrane* (arrowheads), *whereas cells piled up within the duct do not exhibit collagen VII staining* (arrow, *compare with* D, arrow). (G–I) *Invasive carcinoma* (G), *outlined by arrowheads, does not have a BM* (H) *or express collagen VII* (I). *Ln, laminin; C VII, collagen VII; L, lumen.*

antibody-detectable perinuclear intracellular collagen VII, whereas malignant cells had no immunostaining. At about 7 days, malignant cells began to show perinuclear intracellular collagen VII staining, whereas normal cells were already producing basal collagen VII in a secreted pattern.

The M_r 200 protein, on the other hand, continued to be expressed by malignant cells in only a dotted

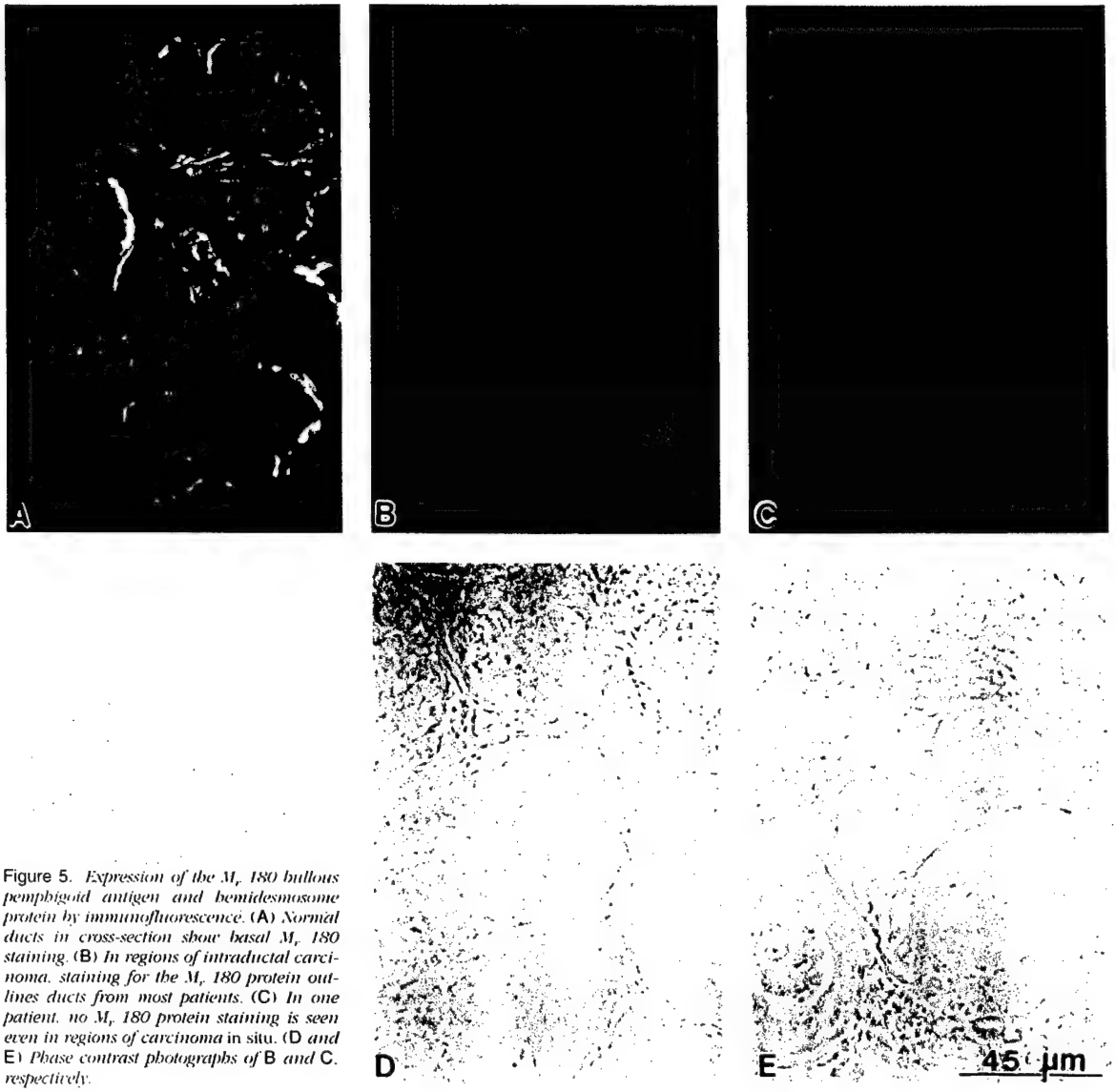

Figure 5. *Expression of the M_r 180 bullous pemphigoid antigen and hemidesmosome protein by immunofluorescence.* (A) *Normal ducts in cross-section show basal M_r 180 staining.* (B) *In regions of intraductal carcinoma, staining for the M_r 180 protein outlines ducts from most patients.* (C) *In one patient, no M_r 180 protein staining is seen even in regions of carcinoma* in situ. (D *and* E) *Phase contrast photographs of* B *and* C, *respectively.*

cytosolic pattern at incubations as long as 30 days (Figure 9). The protein was never expressed basally by malignant cells. The M_r 230 and 180 proteins exhibited lines of basal tick-marks in malignant cells just as in normal cells and with the same time course (Figure 10). As reported previously, the β4 integrin subunit was also expressed identically in normal and malignant cells, whereas the α6 subunit was seen in only ≈30% of malignant cells in culture.[5]

Hemidesmosome Assembly and Regulation

Because normal HMEC formed HD in culture, we used them as a model to study HD assembly. In time courses of HD protein expression as determined by IF, all but one protein were seen intracellularly within 24 hours of plating, but a mature basal plaque-like distribution of proteins was seen with only α6 and the M_r 180 protein (Table 1). Other proteins achieved their mature localization in a specific order (Table 1). HD were seen at the EM level for the first time at 2 weeks.

Because HMEC are highly mobile cells at low cell densities, and HD are structures involved in stable attachment, we wondered whether assembly might be affected by cell density. In fact, localization of the M_r 230 and 200 proteins, β4, and collagen VII were highly dependent on confluence (Table 2). At 14 days in the majority of cells studied when they were at 25% confluence or less, the M_r

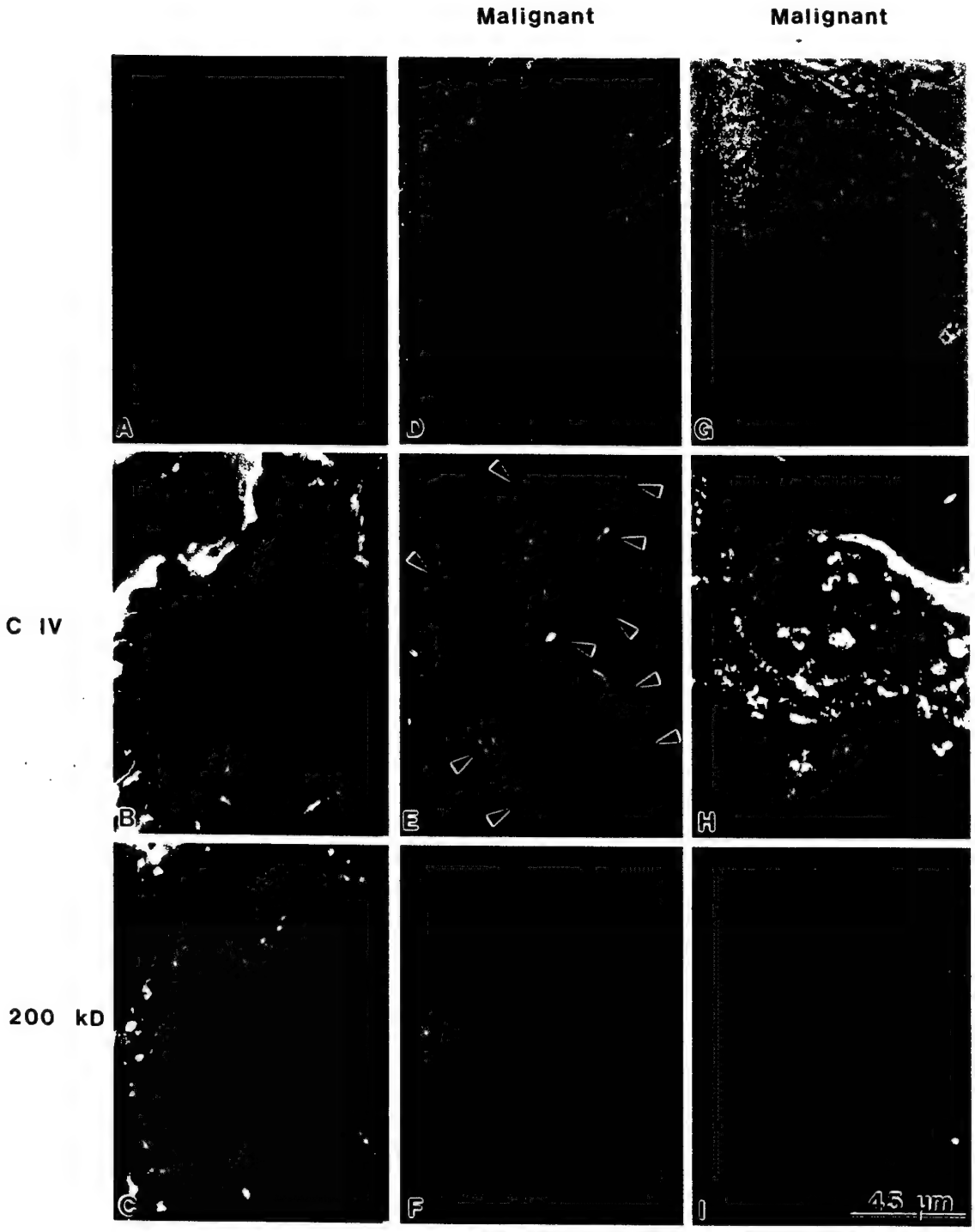

Figure 6. *Expression of the M_r 200 HD protein does not follow the BM by immunofluorescence.* (A) *Negative control for immunofluorescent staining.* (B, C) *A normal duct in cross section has a BM illustrated by collagen IV staining* (B) *and expresses basolateral M_r 200 protein* (C). (D–F) *An intraductal carcinoma in longitudinal section* (D) *has a basement membrane* (E, arrowheads), *but no M_r 200 protein staining* (F). (G–I) *Invasive carcinoma* (G) *exhibits intracellular BM protein staining* (H), *and faint M_r 200 protein staining surrounding each cell* (I). *C IV, collagen IV.*

230 and 200 proteins and collagen VII were localized intracellularly, and β4 was not present, whereas at >40% confluence the four proteins were all present and basally located. On the other hand, α6 and M_r 180 protein localization were insensitive to cell density; these proteins were basally located in all cells at 14 days regardless of confluence.

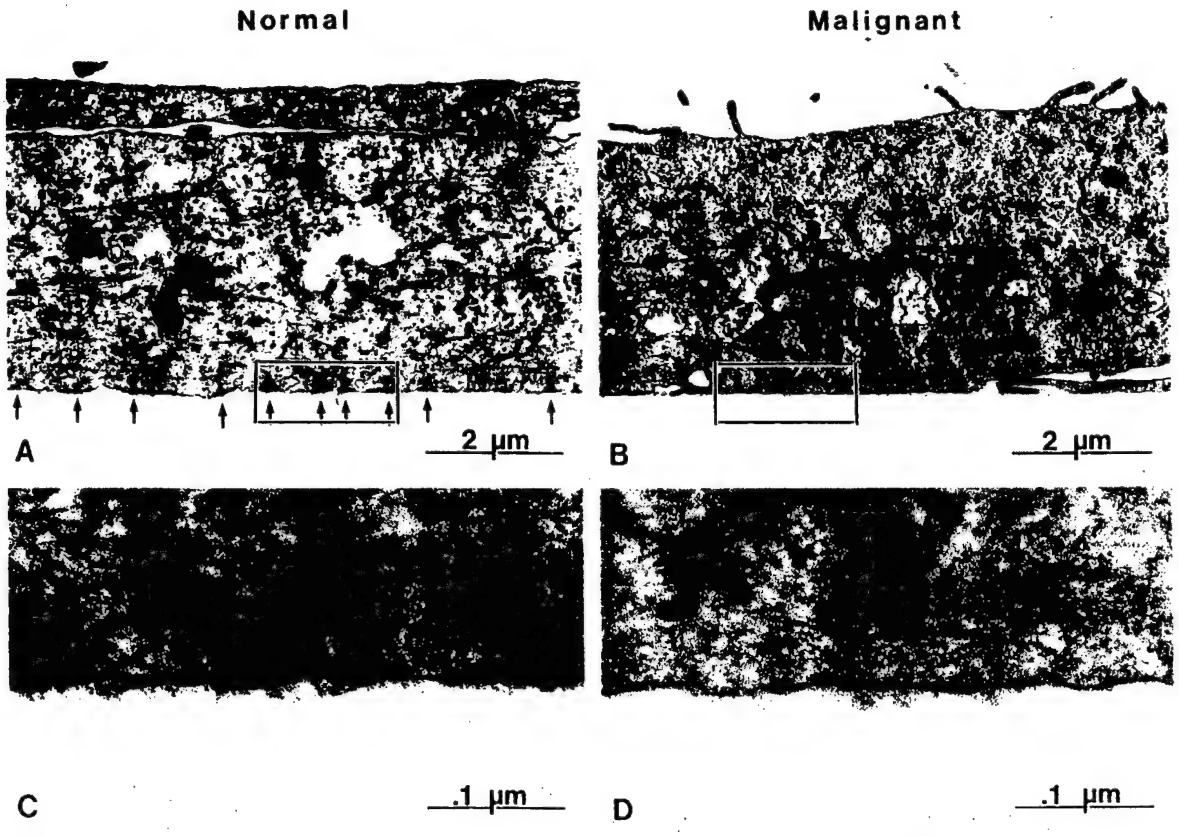

Figure 7. *Electron microscopy of normal and malignant HMEC in culture.* (A) *A normal cell has basal HD* (arrows). (B) *A malignant cell has no HD.* (C) *Higher magnification of box in* A. (D) *Higher magnification of box in* B.

Cell proliferation also influenced HD assembly. We compared protein expression between three cell strains with rapid doubling rates and three cell strains with slow doubling rates by plating cell concentrations that would result in >50% confluence at 2 weeks. Rapidly proliferating cells did not localize the M_r 230 and 200 proteins, β4, or collagen VII basally after the usual 2 weeks, whereas slower growing cells did (Table 3). α6 and M_r 180 protein localization were independent of doubling time as they were of cell density. Even rapidly proliferating cells promptly localized all expected proteins when they reached confluence.

Because motility and proliferation, which appeared to delay HD protein expression, can reflect a dedifferentiated phenotype, we wondered if differentiation might conversely stimulate HD assembly. We plated normal HMEC on Matrigel, a BM-like substance that promotes HMEC morphogenesis and functional differentiation.[30, 38] Electron-dense HD were seen by EM at the bases of cells in fully formed three-dimensional ductlike structures (Figure 11), but not in cells on Matrigel that were still migrating to form these structures (not shown). The process of duct formation was complete at between 14 and 24 days. Likewise, HD were first seen between 14 and 24 days whenever duct formation was complete. Thus HD formation on matrigel appeared to be differentiation-dependent rather than time-dependent.

Finally, because expression of HD in malignant cells *in vivo* appeared to correlate with the presence of BM proteins, we wondered whether ECM might actually stimulate HD assembly. For this purpose we used the laminin-5-rich matrix produced by 804G cells and previously shown to induce rapid HD formation in skin cells.[31] Normal HMEC plated on this matrix formed electron-dense HD within 24 hours instead of the usual 2 weeks (Figure 12). This ECM does not promote HMEC morphogenesis.

Discussion

In previous detailed EM studies of the breast, HD were noted at the BM in basal cells.[23–28] This information seems to have gone relatively unnoticed in the HD literature, where HD continued largely to be discussed as characteristic of stratified epithelia. In addition, it was not clear from past studies whether HD were unique to breast myoepithelial cells or used by all breast epithelial cells to attach to the BM.

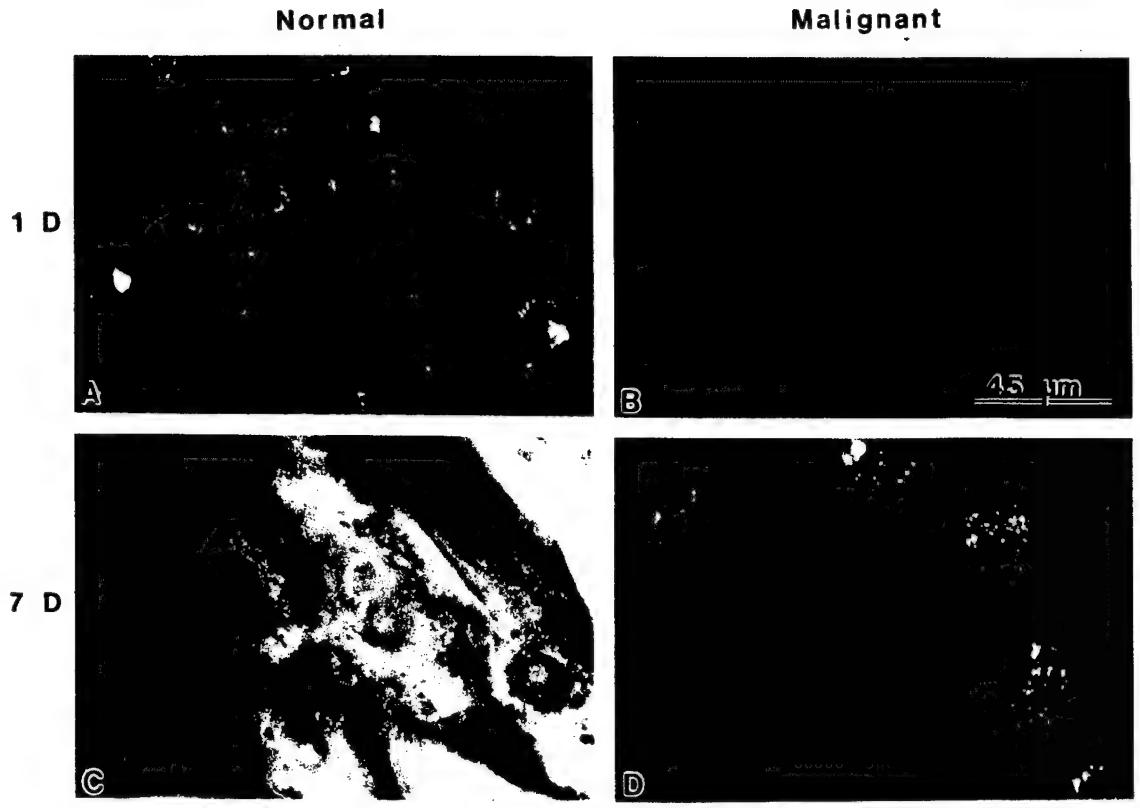

Figure 8. *Delayed expression of the anchoring fibril protein, collagen VII, in cultured malignant HMEC by IF.* (A) *Normal cultured HMEC at 1 day show an intracellular staining pattern for collagen VII.* (B) *Malignant HMEC do not express collagen VII at 1 day.* (C) *By 7 days normal cells stain for collagen VII at the base of cells in a secreted pattern.* (D) *At 7 days malignant cells show an intracellular staining pattern for collagen VII.*

In this study we clearly demonstrate that HD are found where all breast cells, whether myoepithelial or luminal, are in apposition to the BM. This strengthens the data adding breast epithelial cells to the expanding list of cell types that express HD, which now includes stratified epithelia such as skin, cornea, and esophagus[10, 39]; complex epithelia such as trachea, thymus, and transitional epithelia of the urinary bladder[10]; glandular epithelia such as apocrine and salivary glands[26, 10]; and even the simple epithelium of the amnion.[40]

We would like to emphasize that we noted that luminal cells as well as myoepithelial cells contained HD. Because breast carcinoma cells tend to differentiate toward a luminal phenotype in, eg, expression of cytokeratins and actins, it is important to note that absence of HD from invading breast carcinoma cells does not merely represent loss of surrounding myoepithelial cells, but a clear downregulation of HD expression. Recently Clermont et al[41] studied collagen VII anchoring fibrils in the rat breast and also emphasized that both luminal and myoepithelial cells contain abundant HD *in vivo*.

We also determined that normal breast epithelium expressed at least six of the known HD protein components, suggesting that HD have a similar structure and play a similar role in breast as they do in skin and other stratified tissues.

HD are probably involved more in stable rather than motile adhesion. For example, $\alpha 6\beta 4$ was found only in nonmotile keratinocytes in culture,[15] and HD are downregulated in epithelial cells that become motile to fill in a wound (see, eg, refs. 36, 42).

That HD are in fact adhesive structures used by normal epithelia to adhere to BM is suggested by wound healing studies in which reepithelialized cornea can be easily lifted off until BM and HD have formed.[43] Further, the epithelium releases the stroma as a sheet in the genetic blistering diseases dystrophic epidermolysis bullosa, in which anchoring fibrils are congenitally absent,[44, 45] and lethal junctional epidermolysis bullosa, in which HD are abnormal[46]; and in the acquired diseases epidermolysis bullosa aquisita and bullous pemphigoid, in which autoantibodies to collagen VII and to the M_r 230 and 180 HD components, respectively, are found.[47, 48] Also, experimental addition of blocking antibodies to HD proteins either *in vivo* or in culture causes loss of epithelial adhesion.[42]

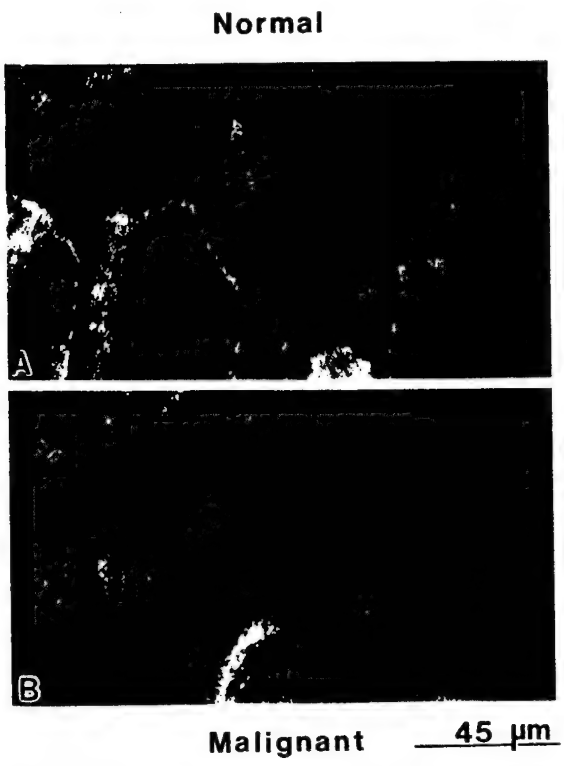

Figure 9. *Aberrant expression of the M_r 200 HD protein in cultured malignant HMEC by IF.* (A) *Normal cells express the M_r 200 protein in rows of basal tick-mark-shaped plaques.* (B) *Malignant cells show a dotted intracellular M_r 200 protein staining pattern.*

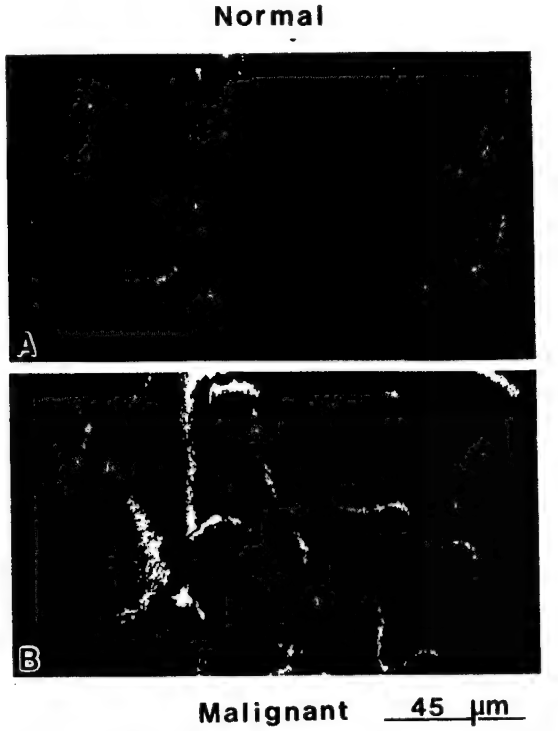

Figure 10. *Identical expression of the M_r 180 HD protein in normal and malignant cultured HMEC. Both normal* (A) *and malignant* (B) *cells express the M_r 180 protein in rows of basal tick-mark-shaped plaques*

The lack of HD in invasive breast carcinoma seen in this study may allow such cells to become less adherent to the BM. During branching morphogenesis that occurs in the breast during embryogenesis, cells in the penetrating endbud lose expression of HD when they invade the stroma and branch into new ducts.[27, 41]

Interestingly, all tumors downregulated the same HD proteins at the same locations regardless of their stage or grade. There are two possible reasons for this. 1) Certain steps in dedifferentiation may typically occur in the same pattern. This apparent pattern relates to the normal pattern of assembly we saw *in vitro.* The proteins that assembled early (M_r 180, α6, collagen VII, and also M_r 230) appear to be coregulated in carcinoma with BM, and may be the proteins that receive a signal from the BM to nucleate HD formation in the normal breast. In fact, α6 and M_r 180 are transmembrane proteins that probably play a role as ECM receptors. On the other hand, M_r 200 and β4 were lost in carcinoma independent of BM and were among the last to be expressed in normal HD formation. Additionally, it was the late proteins that were apparently dependent on motility and proliferative rate for their assembly into HD. 2) Our sample size and exclusive use of ductal carcinoma may have biased our results; and it would be interesting in future studies to compare tumors of different types (e.g., lobular, mucinous, ductal), stages, and grades in sample size with sufficient statistical power to detect differences between subgroups.

Another interesting observation in this study regards HD expression in intraductal carcinoma *in vivo.* We noted HD in the basal cells by EM; however, by IF there were many regions in which the M_r 200 protein and β4 integrin were not expressed by basal cells. This suggests that even in intraductal carcinoma, the apparently ultrastructurally normal HD may actually be abnormal, and could be functionally impaired. Cells piled up within ducts and away from the BM in carcinoma *in situ* had no HD and lacked staining for component proteins. Abnormal downregulation of HD may allow these malignant cells to leave the BM. Alternately, if the BM regulates HD expression, cells that have left the BM with HD intact may then downregulate HD expression as the normal response to a loss of contact with the BM. This brings up the cause and effect question of HD and BM expression: does the BM regulate HD expression or vice versa?

Our *in vitro* results suggest that ECM stimulates HD formation in HMEC. HD formation was accelerated from the 14 days seen on glass to one day by

Table 1. *Time Course of Expression and Localization of HD Proteins and Electron-Dense HD in Normal HMEC in Culture*

	180 kd	α_6	Collagen VII	230 kd	200 kd	β_4	HD visible by EM
Intracellular localization (days after plating)	–*	–	1	1	1	–	–
Mature basal localization (days after plating)	1	1	5–7	10	10	14	14

Average of three experiments using four cell strains. Cells were >50% confluent and of average doubling time. Please refer to text for description of HD proteins.
* = Lack of intracellular localization, these proteins were found at their mature basal location without apparent previous intracellular localization.

Table 2. *Dependence of Basal Localization of HD Proteins in Normal HMEC in Culture on Cell Density*

Plating density (no. cells/35 mm^2)	Final confluence (visual estimates at 14 days)	% of cells/well with basal distribution of 230 kd, 200 kd, collagen VII and β_4	% of cells/well with basal distribution of 180 kd and α_6
5×10^3	5–10%	5 ± 5	100
10^4	15–25%	45 ± 15	100
5×10^4	40–75%	100	100

Average of two experiments using three cell strains. Numbers are mean ± SD. Cells were of average doubling time.

culture on 804G cell matrix. Although a similar effect of matrix on HD assembly was previously noted when epithelial cell lines that did not form HD on plastic were able to make them on collagen I, the impact of 804G matrix is much more rapid.[50, 51, 52] In addition, in embryogenesis, expression of BM clearly precedes HD expression (see, e.g., refs. 53–55). However, most wound healing studies show that in epithelial cells that have migrated to fill a wound, HD reform before or simultaneously with BM.[36, 42, 56–61] In fact, BM initially reforms discontinuously beneath HD as if the HD nucleate BM formation.[44, 62, 63]

It is not clear which of these models best reflects the situation in the normal breast or in breast cancer. In our study there appeared to be a correlation between localization of a cell at the BM and its expression of HD. Therefore the BM could be regulating HD expression. However, some cells at the BM did not express every HD protein, and some cells away from the BM did express some HD proteins. We therefore believe that although lack of HD in invasive breast cancer could be due to a loss of BM, some HD proteins are probably downregulated independently of the BM. In addition, Wetzels et al[64] found, as we did, that collagen VII was lost from almost all (94 of 97) invasive ductal carcinomas, but 13 of these retained staining for BM proteins, suggesting that loss of this HD protein was not a result of loss of BM protein.

It is also possible that the invasive phenotype is an expression of dedifferentiation in general such that both HD and BM protein expression are downregulated by a preceding dedifferentiation event. Such dedifferentiation is seen in wound healing when ep-

Table 3. *Dependence of Basal Localization of HD Proteins in Normal HMEC in Culture on Rate of Proliferation*

Doubling time (days)	% of cells with basal localization of 230 kd, 200 kd, collagen VII, and β_4 at 2 weeks	% of cells with basal localization of 180 kd and α_6 at 2 weeks
3	45 ± 15	100
10	95 ± 5	100

Three cell strains with shorter doubling times (3 days) were compared with three cell strains with longer doubling times (10 days) in three experiments. Numbers are mean ± SD.

Figure 11. *EM of normal HMEC cultured on Matrigel. Cells were grown on Matrigel BM-like substance for 20 days. During this time they underwent differentiation into three-dimensional ductlike structures and assembled HD* (arrows), *which were associated with intermediate filaments* (arrowheads).

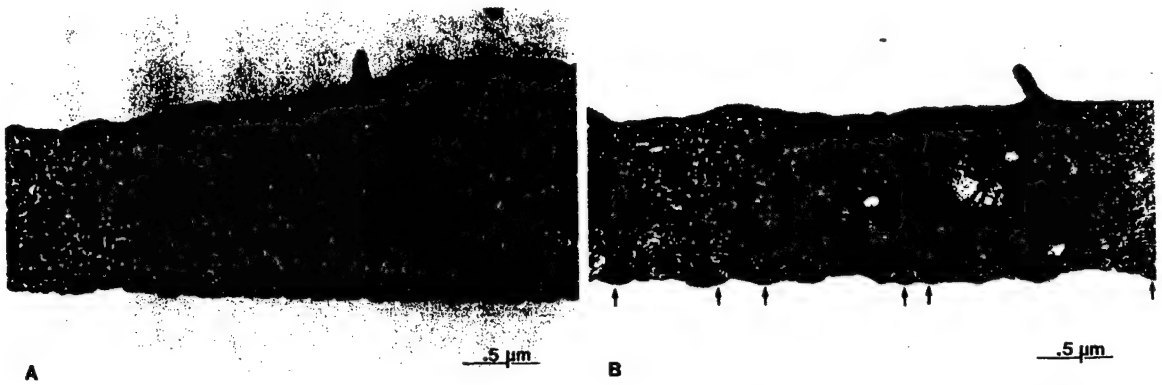

Figure 12. *EM of normal HMEC cultured on 804G matrix.* (A) *HMEC grown on glass coverslips for 24 hours have no HD.* (B) *HMEC grown on laminin-rich 804G matrix for 24 hours contain HD* (arrowheads).

ithelial cells migrate over stroma to reepithelialize denuded regions, and HD and BM proteins are decreased in expression[57, 54] or at least relocated.[42, 65] In addition, HD formation appears to be linked to differentiation events in HMEC, as normal cells on Matrigel formed HD in a manner temporally linked with the differentiation that occurred on that matrix.

With respect to our observations on HD formation in culture, most groups that have reported previously on cells cultured on plastic or glass have seen either no HD by EM, or "prehemidesmosomes" or "immature hemidesmosomes" (reviewed in refs. 11, 65, 66). Recently, however, a few rat and bovine cell lines able to make mature HD have been reported (see, e.g., refs. 10, 66, 67). In this paper we report that our normal primary human cells form HD in culture containing the anticipated protein components. This suggested these breast epithelial cell strains as good models for the study of HD formation.

In time course studies of HD protein expression, most HD proteins were already being made as soon as cells were attached enough to be stained, but they were not all basally located into mature plaques, and EM-recognizable HD were not made until 2 weeks in culture. The order of assembly into mature plaques appears to be α6 and M_r 180 at day 1, then collagen VII, M_r 200 and 230, and finally β4 at the time of final assembly. Kurpakus et al[35, 36, 42] have outlined the order of HD protein assembly in a wound healing tissue culture model, and they also see early appearance of α6, but accompanied by β4. Other HD proteins assemble in a somewhat variable order.[35, 36, 42] It is somewhat surprising that α6 was expressed before β4 in our model, as these two integrin subunits pair to form one integrin molecule in HD. However, the α6 subunit has two possible partners, β1 and β4, to form two different integrins. We see expression of the β1 subunit simultaneous to α6 on day one,[5] but colocalization by IF of α6 and β4 by day 14.[5]

Except for the first two proteins to be localized basally, α6 and M_r 180, the HD proteins studied remained intracellular while cells were mobile or highly proliferative. This suggests that these cells are able to couple HD localization to machineries of cell cycle and motility. This is consistent with data in the skin in which epithelial cells proliferating and migrating to cover a wound contain HD proteins intracellularly only, but assemble basal HD when migration and proliferation are complete.

Several of the parameters that appear to regulate HD expression by normal HMEC are factors perturbed in carcinoma: motility, proliferative rate, and BM synthesis. In fact, these interrelationships may play a role in the downregulation of HD we observed in malignancy. Several malignant breast cell strains in culture lacked HD by EM; however, some malignant cells in culture did express HD. This heterogeneity probably reflects the heterogeneity of the tissues from which the cells were derived. For example, in cells grown from one tumor, some cells were seen with and others without HD by EM; these cells could represent intraductal and invasive cells, respectively. In the one cell strain where we saw a normal complement of HD, only intraductal cells may have grown; and in the three cell strains with no HD, only invasive cells may be represented. It is also possible that cells derived from higher stage or grade tumors might exhibit the more abnormal phenotype. This could not be determined from the sample size in this study, but warrants further investigation.

Malignant cells in culture had a more normal array of HD protein expression by IF than did malignant cells *in vivo*. As mentioned above, it is possible that the cells grown in culture were not fully representative of those found *in vivo*. For example, if a majority

of the cells in culture were derived from the intraductal portion of carcinomas, a more normal phenotype would be observed.

A more interesting possibility is that HD proteins not expressed *in vivo* can be reexpressed by tumor cells in a different milieu in culture. This suggests that the ability to express HD proteins may not be lost by malignant cells; rather, HD proteins may be downregulated *in vivo* by a program of altered differentiation that is partially reexpressed in culture. This was also suggested by the fact that malignant cells *in vivo* had such a complete change in HD protein expression, when a loss of only one protein might have been expected if the loss were due to a mutation in that gene. It is further supported by the fact that collagen VII, which is eventually expressed basally in malignant cells in culture as in normal cells, exhibits a delayed conversion to the normal phenotype from that of intracellular expression. However, the fact that the M_r 200 protein remained abnormal in culture suggests the possibility of a permanent change in expression of this particular protein. Therefore, both the regulation of HD proteins and some of the proteins themselves may be abnormal in breast cancer. This hypothesis will have to be tested further in subsequent studies.

As mentioned above, malignant breast cells in culture did exhibit some abnormalities of HD protein expression by IF: the M_r 200 protein and collagen VII were seen intracellularly. This localization is reminiscent of wound healing, in which HD proteins are also expressed intracellularly by epithelial cells migrating to close a wound.[68] Some aspects of cancer invasion have been compared with wound healing, and internalization of HD components could be a common mechanism by which epithelial cells become migratory.

In conclusion, in this paper we note the presence of HD in normal HMEC both *in vivo* and in culture containing the expected proteins, and a correlation of increasing downregulation of expression of HD with increasing aggressiveness of tumor cells. Such a correlation has been previously noted in other malignancies and with other adhesion molecules (reviewed in refs. 69–71; see also refs. 72, 73 for recent updates). We expand their data to include HD and breast cancer, and suggest that normal HMEC may use HD to maintain their position in the mammary duct, and malignant cells may use downregulation of HD as a means of escape from usual tissue architectural restraints.

Further, our manipulations of cells in culture together with *in vivo* data suggest a model for HD protein regulation. Early HD protein localization may be signaled by the extracellular matrix and regulated by it, whereas localization of late HD proteins (those usually localized to cell bases later) appear to be coupled to motility and proliferation. Both sets of proteins are affected in malignancy in which ECM, motility, and proliferation are all abnormal. Because HD expression appears to be linked to differentiation, this may reflect a program of dedifferentiation by malignant cells *in vivo* that may be able to be partially reversed under certain circumstances (e.g., in culture) suggesting that these malignant changes may be a combination of genetic and epigenetic events.

Acknowledgments

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The role of laminin-5 and its receptors in mammary epithelial cell branching morphogenesis

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SUMMARY

In vivo, normal mammary epithelial cells utilize hemidesmosome attachment devices to adhere to stroma. However, analyses of a potential role for hemidesmosomes and their components in mammary epithelial tissue morphogenesis have never been attempted. MCF-10A cells are a spontaneously immortalized line derived from mammary epithelium and possess a number of characteristics of normal mammary epithelial cells including expression of hemidesmosomal associated proteins such as the two bullous pemphigoid antigens, α6β4 integrin and its ligand laminin-5. More importantly, MCF-10A cells readily assemble mature hemidesmosomes when plated onto uncoated substrates. When maintained on matrigel, like their normal breast epithelial cell counterparts, MCF-10A cells undergo a branching morphogenesis and assemble hemidesmosomes at sites of cell-matrigel interaction. Function blocking antibodies specific for human laminin-5 and the α subunits of its two known receptors (α3β1 and α6β4 integrin) not only inhibit hemidesmosome assembly by MCF-10A cells but also impede branching morphogenesis induced by matrigel. Our results imply that the hemidesmosome, in particular those subunits comprising its laminin-5/integrin 'backbone', play an important role in morphogenetic events. We discuss these results in light of recent evidence that hemidesmosomes are sites involved in signal transduction.

Key words: Hemidesmosome, Integrin, Laminin

INTRODUCTION

Extracellular matrix plays a crucial role in determining the morphogenesis of a number of epithelial tissue types (Hay, 1993). One of the most dramatic examples of this phenomenon is the regulation of mammary epithelium phenotype by elements of basement membranes derived from the Engelbreth-Holm-Swarm tumor (matrigel) (Bissell and Ram, 1989; Barcellos-Hoff et al., 1989; Blum et al., 1989; Lin and Bissell, 1993). Indeed, mouse mammary epithelial cells assemble into structures remarkably similar to alveoli of lactating mammary glands and produce milk proteins when maintained in matrigel (reviewed by Lin and Bissell, 1993).

Compared with the rodent system, analyses of morphogenesis of human mammary epithelial cells have progressed more slowly, in part because of difficulties in maintaining cultures of primary human cells. This problem has been partially alleviated by the development of media for the culture of primary human mammary epithelial (HMEC) cells although establishment of primary cultures remains problematic (Stampfer, 1985; Bergstraesser and Weitzman, 1993). One alternative is the use of continuous human mammary epithelial cell lines such as MCF-10A (Soule et al., 1990). Indeed, a model for the study of mammary epithelial cell morphogenesis using MCF-10A cells has recently been described (Howlett et al., 1995).

It has now been shown that laminin-1 is the matrix component of matrigel which regulates morphogenesis as well as milk protein expression of mouse mammary epithelial cells in vitro (Streuli et al., 1995). Furthermore, the domain responsible for such regulation resides in the so-called E3 fragment of laminin-1 and is located towards the carboxy terminus of the α1 subunit of the heterotrimer (Streuli et al., 1995). Laminin-1, via its cell surface receptors, is believed to establish polarity of mammary epithelial cells, a process which is an essential prerequisite to cell differentiation (Streuli et al., 1995). However, following polarization, it is hypothesized that epithelial cells modulate their own microenvironment by producing additional basement membrane components (Bissell and Ram, 1989). The latter could include a number of laminins since laminin-1 is only one of several laminin isoforms which occur in intact basement membranes (Timpl and Brown, 1994). For example, laminin-5 is widely distributed in the basement membranes of epithelial tissues, including the mammary gland, as we show here (Verrando et al., 1987; Rousselle et al., 1991; Carter et al., 1991; Kallunki et al., 1992; Timpl and Brown, 1994). Do these endogenously secreted basement membrane elements play a role in mammary epithelial morphogenesis? To answer this question, we have analyzed the function of laminin-5 in an in vitro model of mammary epithelial morphogenesis using MCF-10A cells. These cells undergo branching morphogenesis i.e. assemble a highly anastomosed multicellular network, when cultured on matrigel. We show that matrigel-induced differentiation of MCF-10A cells is inhibited by function blocking laminin-5 antibodies as well as antibodies against two distinct laminin-5 receptors. Since laminin-5 is a component of certain cell-matrix junctions called

hemidesmosomes and MCF-10A cells assemble hemidesmosomes in vitro, we discuss the possibility of signaling events transduced by these complex morphological entities.

MATERIALS AND METHODS

Cell culture

MCF-10A cells were obtained from the American Tissue Culture Collection (Rockville, MD) and were maintained in a 1:1 mix of DME and Ham's F12 media supplemented with 5% equine serum, 0.01 mg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone. SCC12 cells were maintained in a serum free growth medium (Medium 154; Cascade Biologics, Inc., Portland, OR).

For our morphogenesis assays, matrigel was purchased from Collaborative Biomedical Products (Bedford, MA) and was coated onto plastic dishes at approximately 15 mg/ml. The dishes were subsequently incubated at 37°C for 30 minutes prior to addition of cells. In some instances, cells were mixed with liquid matrigel at 4°C. The cell/matrigel mix was then pipetted onto plastic and allowed to gel at 37°C.

Antibodies

GB3, a mouse monoclonal antibody which recognizes the γ chain of human laminin-5, was obtained from Harlan Bioproducts for Science, Inc., Indianapolis, IN (Verrando et al., 1987; Matsui et al., 1995). Mouse monoclonal antibody, clone 17, specific for the β chain of laminin-5 was purchased from Transduction Laboratories (Lexington, KY). Dr William Carter, Fred Hutchinson Cancer Research Center generously provided C2-9, a function blocking mouse monoclonal antibody specific for the α3 chain of laminin-5 and P1E1, a non-function blocking antibody which also recognizes the α3 chain of human laminin-5 (Xia et al., 1996). We used P1E1 as a control IgG in some of our antibody inhibition studies. The rabbit serum J17, against BP180 and the mouse monoclonal antibody 10C5, against BP230, have been described elsewhere (Hopkinson et al., 1992; Hopkinson and Jones, 1994). GoH3, a rat monoclonal which recognizes the α6 integrin subunit, was purchased from Immunotech (Westbrook, ME). P1B5 and 3E1, mouse monoclonal antibodies which recognize the α3 integrin and β4 integrin subunits, respectively, were purchased from Gibco BRL (Gaithersburg, MD). Rabbit sera 6945 and 6845, against β4 integrin and the 'light' chain of the α6 integrin subunit, respectively, were kindly provided by Dr Vito Quaranta, Scripps Institute (Tamura et al., 1990).

Immunofluorescence

MCF-10A cells, maintained on glass coverslips, were either permeabilized in acetone at –20°C for 2 minutes and air dried thoroughly, or, for integrin localization, were first fixed for 5 minutes in 3.7% formaldehyde, washed thoroughly in PBS, and then permeabilized in acetone at –20°C for 2 minutes prior to air drying. Cells maintained in matrigel were prepared for immunofluorescence analyses by first fixing them for 10 minutes in 3.7% formaldehyde. After washing thoroughly in PBS, they were permeabilized with 0.5% Triton X-100 in PBS at 4°C for 10 minutes and then washed once again in PBS. Preparations were incubated with primary antibody diluted in PBS at 37°C in a humid chamber for 1 hour, washed 3 times in PBS, and incubated with an appropriate fluorochrome-conjugated secondary antibody for a further 1 hour at 37°C.

For frozen tissue sections, normal human breast tissue from a reduction mammoplasty was received from the Cooperative Human Tissue Network (Columbus, OH). Tissue was snap frozen in liquid nitrogen and embedded in Tissue-Tek OCT compound (Miles Laboratory, Elkhart, IN). Sections (10 μm) of the frozen tissue were prepared and mounted on poly-L-lysine coated microscope slides. Sections were fixed for 5 minutes in –20°C acetone, air-dried thoroughly, and stained for immunofluorescence as above.

Fluorescence specimens were visualized using a Zeiss LSM10 laser scanning confocal microscope (Zeiss Inc., Thornwood, NY). Images were stored on Sony optical discs and printed on a Tektronix printer (Tektronix, Wilsonville, OR).

Protein preparations, SDS-PAGE and western immunoblotting

Confluent cell cultures were solubilized in sample buffer consisting of 8 M urea, 1% sodium dodecyl sulfate (SDS) in 10 mM Tris-HCl, pH 6.8, and 15% β-mercaptoethanol. DNA was sheared by sonication using a 50 W Ultrasonic Processor (Vibracell Sonics and Materials Inc., Danbury, CT). Matrix of MCF-10A cells was prepared according to Gospodarowicz (1984) and solubilized in sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and processed for immunoblotting according to the method of Zackroff et al. (1984).

Immunoprecipitation

Subconfluent dishes of MCF-10A cells were radiolabeled overnight with 50 μCi/ml of ^{35}S-PRO-MIX cell label (Amersham Corp., Arlington Heights, IL). Conditioned medium of the labeled MCF-10A cells was collected and then pre-cleared by incubation with Protein G-Sepharose beads (Gibco BRL, Gaithersburg, MD) for one hour at 4°C. After centrifugation, monoclonal antibodies were added to the supernatant and the mix was then incubated for 1 hour at 4°C. Protein G-Sepharose beads were added and the tubes incubated for an additional hour at 4°C. Beads were collected by centrifugation and washed 5 times in TBS (10 mM Tris-HCl, pH 7.4, 145 mM NaCl and 1 mM PMSF) containing 1% Triton X-100. Proteins eluted from the beads in sample buffer were processed for SDS-PAGE/autoradiography as well as immunoblotting.

Electron microscopy

Cells maintained on tissue culture plastic or on matrigel were fixed for a minimum of 30 minutes in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After being washed three times in 0.1 M sodium cacodylate buffer, cells were post-fixed in 1% OsO_4 containing 0.8% potassium ferricyanide. Preparations were subsequently stained with uranyl acetate, dehydrated in ethanol, and embedded in Epon-Araldite resin (Tousimis Corp., Rockville, MD). Thin sections of embedded material were stained with lead nitrate and sodium citrate and viewed at 60 kV in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA).

RESULTS

MCF-10A cells express laminin-5 as well as hemidesmosomal proteins

MCF-10A cells were maintained on glass coverslips for 24 hours and then processed for immunofluorescence microscopy using monoclonal antibodies against laminin-5. The latter stain in a leopard spot pattern along sites of cell-substrate association as determined by confocal laser scan microscopy (Fig. 1A). Laminin-5 antibody reactivity also occurs along areas of the glass coverslip where there are no apparent cells, suggesting that the MCF-10A cells leave behind 'trails' of laminin-5 as they migrate over their substrate. Since MCF-10A cells are derived from human mammary glands, we also determined whether laminin-5 is a component of breast epithelial basement membranes. Indeed, basement membranes encircling groups of breast epithelial cells show strong reactivity with laminin-5

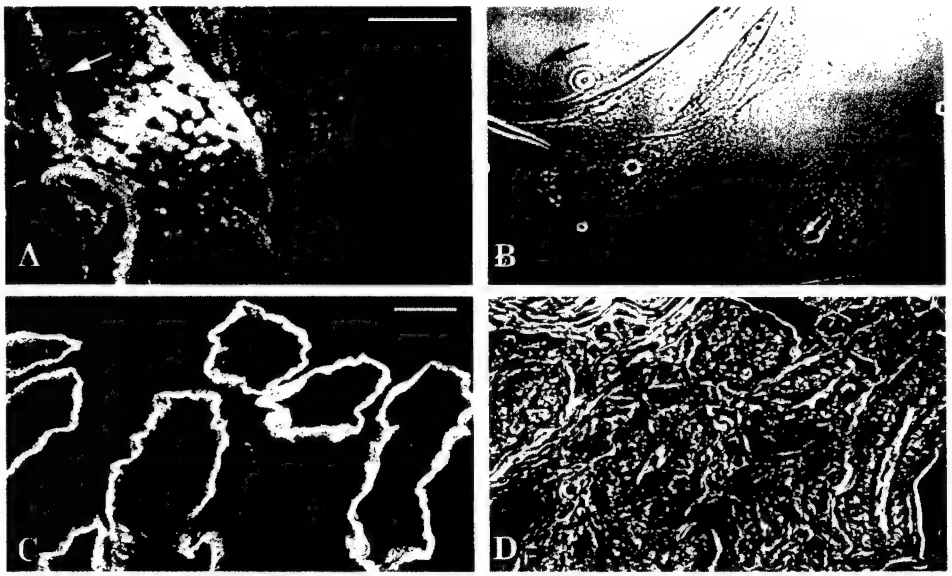

Fig. 1. Laminin-5 is expressed by MCF-10A cells and in human breast tissue. MCF-10A cells were cultured on glass coverslips and processed for indirect immunofluorescence microscopy using the laminin-5 monoclonal antibody (GB3) (A). The cells were viewed by confocal microscopy, the plane of focus being close to the cell-substrate interface. The laminin-5 antibodies stain in a typical leopard spot pattern. The GB3 antibodies also stain areas where there are no apparent cells (arrow). (C) A cryosection of human breast tissue from a reduction mammoplasty was processed for immunofluorescence with GB3 antibodies. These stain the basement membrane zones of islands of epithelial cells. (B and D) Phase contrast images. Bars, 10 μm.

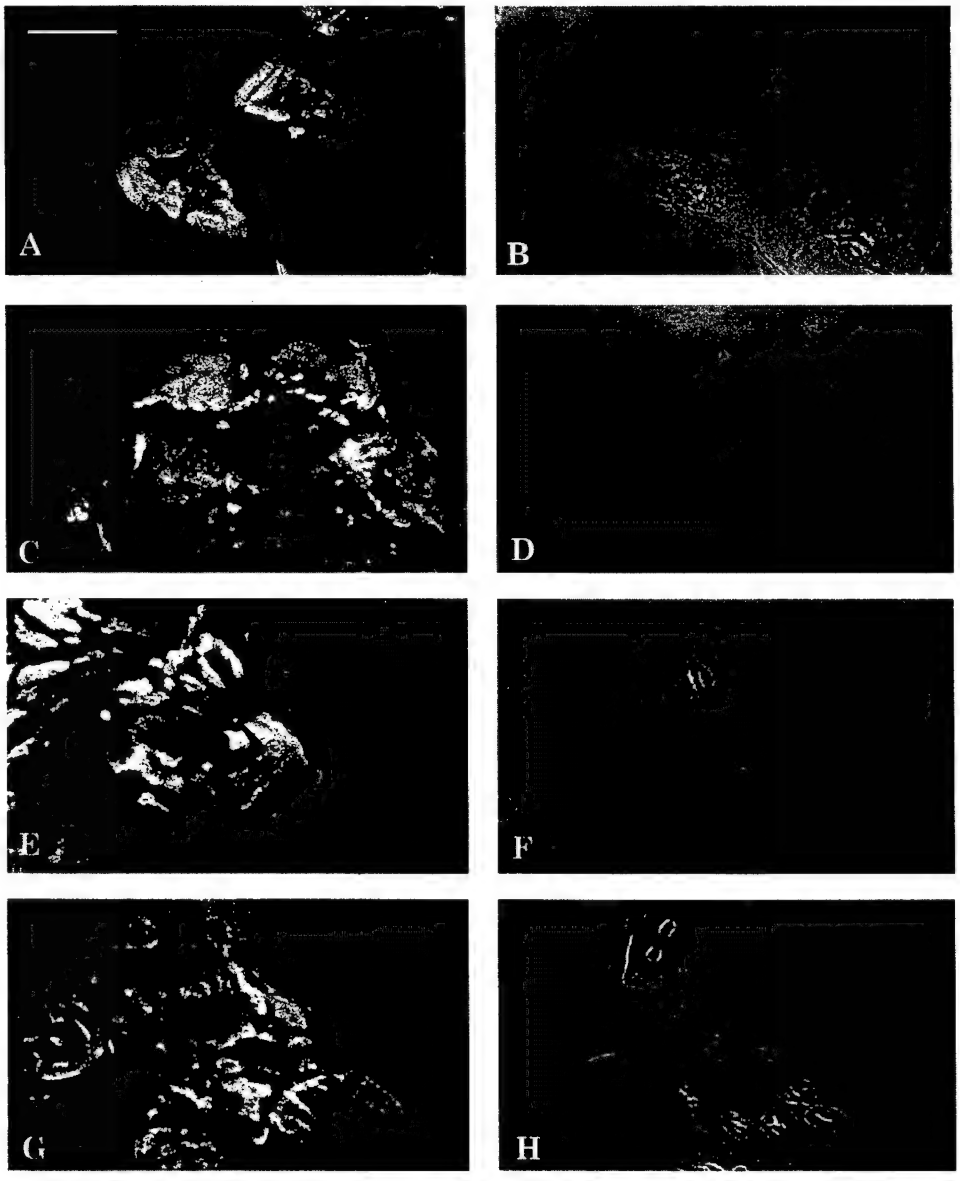

Fig. 2. Hemidesmosomal proteins are expressed by MCF-10A cells as shown by indirect immunofluorescence. MCF-10A cells, maintained on glass coverslips were processed for indirect immunofluorescence microscopy using antibodies specific for BP180 (J17) (A), BP230 (10C5) (C), β4 integrin (3E1) (E), and α6 integrin (GoH3) (G). In all cases the antibodies generate a patchy, leopard spot stain along the region of cell-coverslip interaction. (B,D,F,H) Phase contrast images of the cells. Bar, 10 μm.

antibodies in cryosections of mammary tissue material (Fig. 1C).

In addition to laminin-5, MCF-10A cells, processed for indirect immunofluorescence microscopy, are recognized by antibodies against major components of hemidesmosomes including both bullous pemphigoid antigens (BP180, BP230) as well as the β4 and α6 integrin subunits (Jones et al., 1994; Green and Jones, 1996) (Fig. 2). All of these antibodies generate similar leopard spot staining patterns along the basal aspect of the adherent cells (Fig. 2). This pattern is comparable to that generated by laminin-5 antibodies (Fig. 1A). However, unlike laminin-5, there is an absence of hemidesmosome protein in areas of the glass coverslips devoid of cells (Fig. 2).

Electron microscopic analyses of MCF-10A cells reveals that they assemble hemidesmosome-like structures where they abut their substrates (Fig. 3). These structures possess all of the morphological features of hemidesmosomes observed in mammary epithelial cells in situ i.e. they have triangular shaped, trilayered cytoplasmic plaques (Fig. 3; Jones et al., 1994; Bergstraesser et al., 1995).

To confirm that MCF-10A cells express hemidesmosome components, we have analyzed cell extracts by immunoblotting using antibodies directed against BP180 and BP230, and antisera against β4 integrin and the 'light' chain of α6 integrin (Fig. 4A). These antibodies recognize species of 180, 230, 200 and 30 kDa, respectively (Fig. 4A, lanes 1,3,5 and 7). Furthermore, the MCF-10A hemidesmosomal proteins co-migrate with their epidermal equivalents present in extracts of SCC12 cells (Fig. 4A, lanes 2,4,6 and 8).

MCF-10A cells produce a laminin-5 rich matrix and secrete soluble laminin-5

We have analyzed both the matrix deposited onto substrates by MCF-10A cells as well as MCF-10A conditioned medium for the presence of laminin-5 using a combination of immunoblotting and immunoprecipitation. MCF-10A matrix was prepared according to the procedure of Gospodarowicz (1984). This matrix contains four prominent polypeptides of 155, 135, 100 and 80 kDa and is rich in subunits of laminin-5 as shown by immunoblotting using a monoclonal antibody which recognizes the β2 135 kDa laminin-5 subunit (Fig. 4B). In addition, the 155, 135 and 100 kDa species present in MCF-10A matrix co-migrate with the major polypeptides immunoprecipitated from MCF-10A conditioned medium by two laminin-5 monoclonal antibodies (GB3 and C2-9) (Fig. 4C, lanes 1 and 3). The 135 kDa polypeptides immunopreciptated from MCF-10A conditioned medium by both these anti-laminin-5 monoclonal antibodies are recognized by the β2 chain antibody in immunoblots (Fig. 4C, lanes 2 and 4).

MCF-10A cells undergo branching morphogenesis when plated on matrigel

When MCF-10A cells are embedded into liquid matrigel, which is then allowed to gel, they remain as discrete cellular aggregates ('acini') for 7 days or more regardless of cell concentration (Howlett et al., 1995). In contrast, MCF-10A cells form an interconnected set of tube-like structures, one day after being plated at a concentration of 2.5×10^4 cells/cm^2 on top of matrigel (Fig. 5A). These are similar to the networks of HMECs observed in matrigel and collagen I gels (Bergstraesser et al., 1996; Berdichevsky et al., 1994).

The ability of MCF-10A cells to assemble into tube-like arrays is cell concentration dependent. At cell concentrations of 1.25×10^4/cm^2 or below the MCF-10A cells remain as small aggregates on the matrigel (Fig. 5B). Indeed, they remain in similar aggregates even at 7 days following plating (result not shown).

The tube-like multicellular aggregates of MCF-10A cells in matrigel were processed for confocal immunofluorescence microscopy using antibodies against laminin-5, α6 integrin and α3 integrin (Fig. 6). Both laminin-5 and α6 integrin are concentrated along the edges of the MCF-10A tubes where the cells abut matrigel (Fig. 6A,B). α3 integrin is localized at the

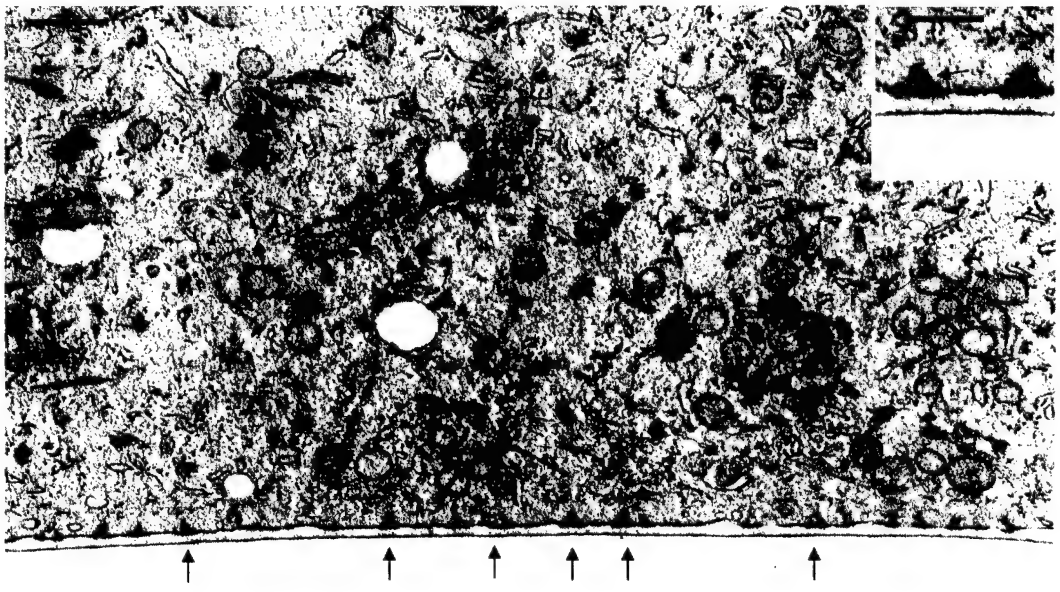

Fig. 3. MCF-10A cells assemble hemidesmosomes when maintained in vitro. This electron micrograph shows a cross section of MCF-10A cells. Arrows indicate numerous electron dense hemidesmosome structures. These possess tripartite cytoplasmic plaques (inset, arrow). Bar, 500 nm (inset, 250 nm).

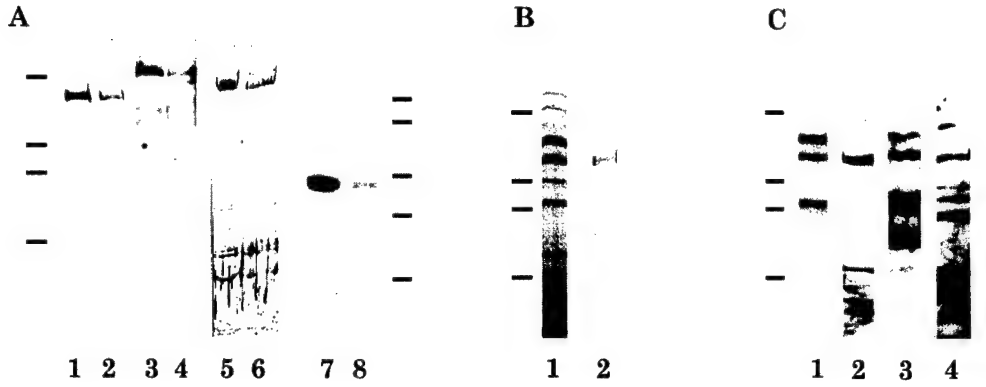

Fig. 4. (A) Hemidesmosomal proteins are expressed by MCF-10A cells as shown by immunoblotting. MCF-10A cell extracts (lanes 1,3,5 and 7) and extracts of SCC12 cells, a keratinocyte line (lanes 2,4,6 and 8) were separated by SDS-PAGE on either 6% (lanes 1-6) or 15% (lanes 7,8) polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antibodies against BP180 (J17, lanes 1,2), BP230 (10C5, lanes 3,4), β4 integrin (6945, lanes 5,6), or the 'light' chain of α6 integrin (6845, lanes 7,8). (B) MCF-10A cells deposit laminin-5 on their substrate. MCF-10A matrix was collected according to the method of Gospodarowicz (1984), processed for SDS-PAGE on a 6% gel, and either silver stained (lane 1) or transferred to nitrocellulose and immunoblotted with a monoclonal antibody (clone 17) against the β chain of laminin-5 (lane 2). In the silver stained preparation, there are prominent polypeptides at 150, 135 and 100 kDa representing the α, β and the γ chains of laminin-5 (lane 1). The 135 kDa protein in this preparation is recognized by the clone 17 antibody (lane 2). (C) MCF-10A secrete laminin-5 into their medium. The medium conditioned by radio-labeled MCF-10A cells was processed for immunoprecipitation using two monoclonal laminin-5 antibodies (GB3, lanes 1,2; C2-9, lanes 3,4). The immunoprecipitated proteins were analyzed by SDS-PAGE/autoradiography (lanes 1 and 3) or prepared for immunoblotting using clone 17 monoclonal antibody against the β chain of laminin-5 (lanes 2 and 4). The laminin-5 antibodies precipitate three major polypeptides of 150, 135 and 100 kDa (lanes 1,3). The 135 kDa protein is recognized by the clone 17 antibody (lanes 2,4). Note that there is some breakdown of the laminin-5 in the C2-9 antibody precipitate (lane 2). This may explain the ladder of proteins recognized by the clone 17 antibody in lane 4. The low molecular mass reactive species in lanes 2 and 4 are due to cross reactivity of the secondary antibody anti-mouse IgG with the immunprecipitated mouse IgG. Bars on the left side of A, B, and C indicate molecular mass standards of 200, 116, 97.4, and 66 kDa. Bars on the right side of A indicate standards of 66, 45, 31, 21.5 and 14.5 kDa. Each lane of the gels was loaded with approximately 10 μg of protein.

latter sites although it is also present at areas of cell-cell contact (Fig. 6C). An IgG control fails to stain the cell population in Fig. 6D.

Antibody inhibition of MCF-10A morphogenesis

We next used an immunological approach to assess the potential role of laminin-5 and its receptors (the integrins α6β4 and α3β1) in matrigel induced branching morphogenesis of MCF-10A cells. For these studies MCF-10A cells were incubated for 15 minutes at 37°C in medium containing either control IgG (50 μg/ml) or in function blocking antibodies directed against α6 integrin (GoH3 at 50 μg/ml), α3 integrin (P1B5 diluted 1:20) and laminin-5 (C2-9 diluted 1:5) (Fig. 7). The cells in the antibody containing medium were plated onto matrigel coated surfaces at 2.5×10^4/cm^2. After 24 hours the cells incubated in control IgG had formed long interconnected tubes whereas there was an obvious inhibition of branching morphogenesis in cultures which had been incubated in the α3 and α6 integrin antibodies as well as those cells incubated with the laminin-5 antibodies (Fig. 7).

We also fixed and processed the antibody treated cells for electron microscopy. We analyzed at least twenty MCF-10A cells in contact with matrigel under each experimental condition (Fig. 8). MCF-10A cells plated onto matrigel in the presence of control IgG assemble hemidesmosomes at sites of cell-matrigel association (Fig. 8A). The latter appear as electron dense structures with extracellular sub-basal dense plates which indicate formation of 'mature' hemidesmosomes (Fig. 8A, inset). In contrast, no hemidesmosomes were observed along regions of cell-matrigel interaction in cultures incubated in function blocking α3 integrin, α6 integrin and laminin-5 antibodies (Fig. 8B-D).

Conclusions

In this study we have shown that MCF-10A cells, an immortalized mammary epithelial cell line, like HMECs, derived from reduction mammoplasties, undergo a branching morphogenesis when maintained on matrigel (Bergstraesser et al., 1996). This phenomenon is highly dependent on cell concentration. We have never observed the formation of tubular arrays when MCF-10A cells are plated onto matrigel at concentrations below 1.25×10^4cells/cm^2. Just a twofold increase

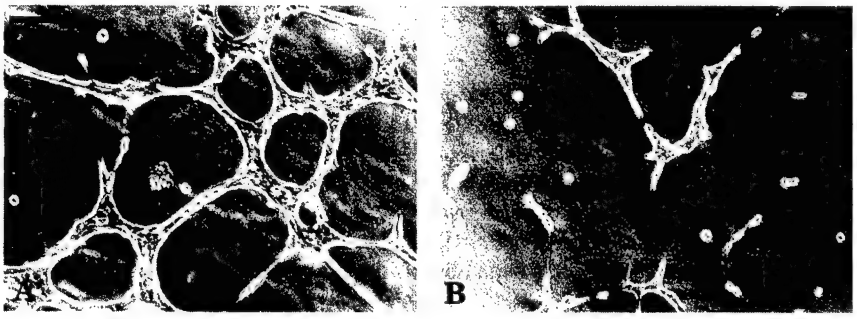

Fig. 5. MCF-10A cells undergo branching morphogenesis on matrigel in a cell concentration dependent manner. 2.5×10^4/cm^2 (A) and 1.25×10^4/cm^2 (B) MCF-10A cells were plated onto matrigel which had been used to coat 35 mm dishes. At 24 hours following plating, the cells in A have undergone a branching morphogenesis while the cells in B appear in small aggregates. Bar, 500 μm.

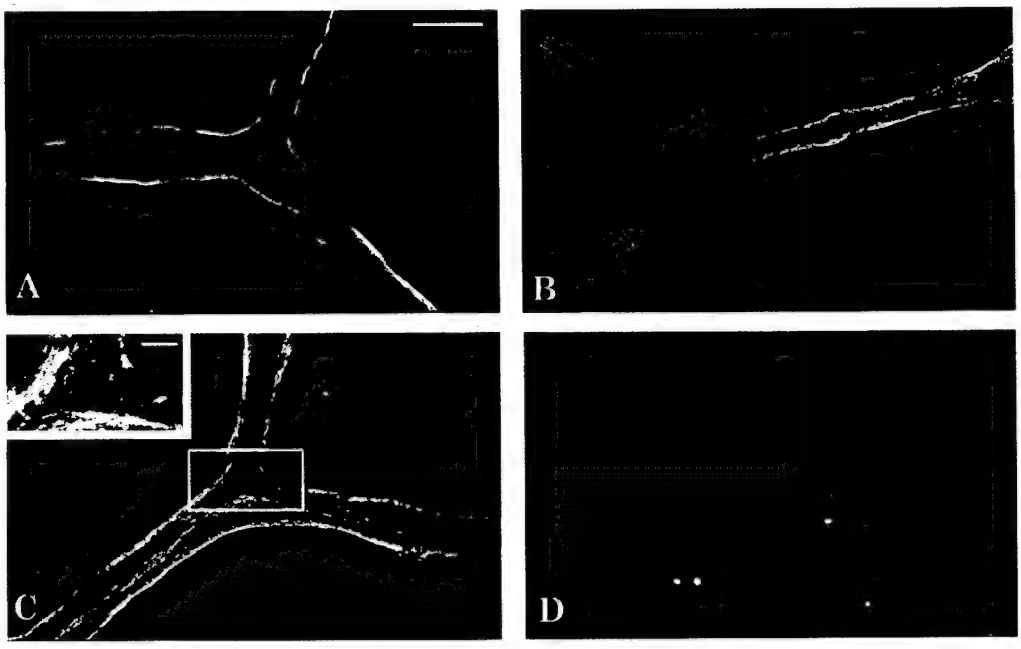

Fig. 6. Laminin-5 and its receptors are expressed by MCF-10A cells undergoing morphogenesis on matrigel. MCF-10A cells maintained on matrigel for 24 hours were processed for indirect confocal immunofluorescence with monoclonal antibodies recognizing laminin-5 (GB3, A), α6 integrin (GoH3, B), α3 integrin (P1B5, C), or an IgG control (D). Note that the antibodies in A,B and C show staining along regions of cell-matrigel interaction. The inset in C is a higher magnification of the boxed area and reveals that α3 integrin occurs at sites of cell-cell as well as cell-matrigel interaction. Bars: (A), 100 μm; (C), 25 μm.

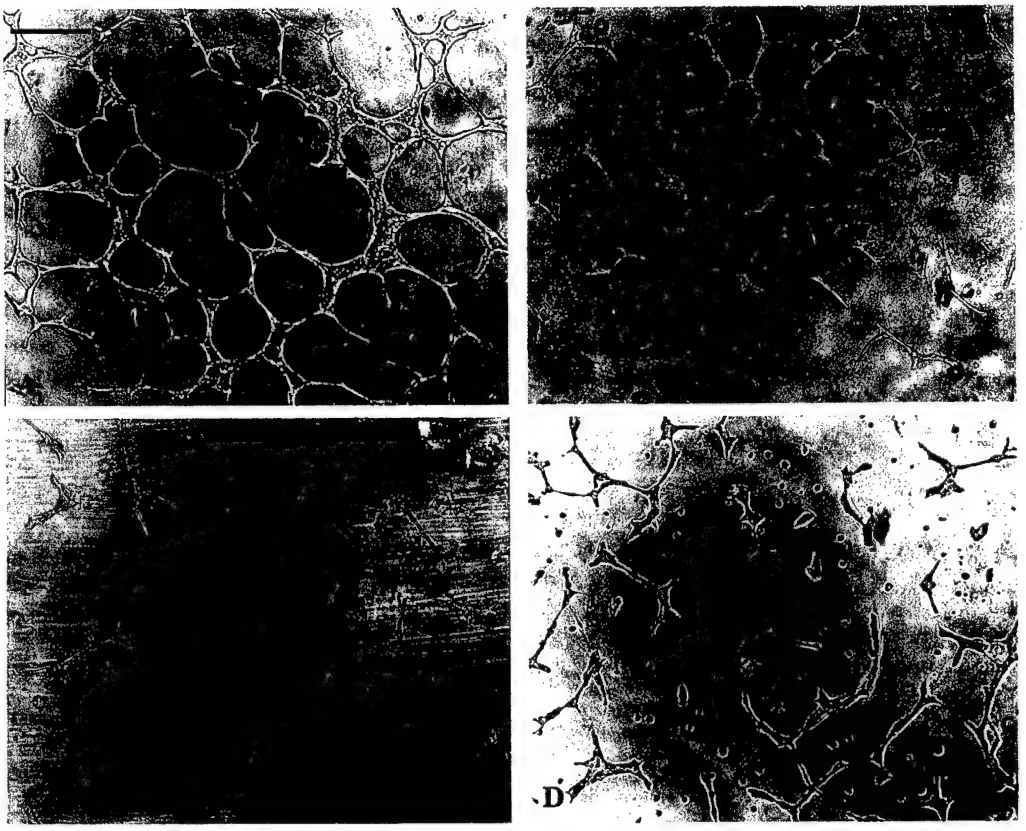

Fig. 7. Branching morphogenesis of MCF-10A cells on matrigel is inhibited by antibodies against laminin-5, α6 integrin and α3 integrin. MCF-10A cells were plated onto matrigel in the presence of control IgG (A), or antibodies which block the function of α6 integrin (GoH3, B), α3 integrin (P1B5, C), or laminin-5 (C2-9, D). After 24 hours, the cells in A appear organized into a highly branched array, while those incubated with blocking antibodies remain either as single cells or in small multicellular clusters (B,C and D). The large dark circle in each of the micrographs is an optical artifact. Bar, 100 μm.

in this cell number is enough to trigger a matrigel induced branching morphogenesis of the MCF-10A cells. Indeed, we find it remarkable that within 1 day of plating onto matrigel, MCF-10A cells assemble into an anastomosing network, organized into a branching pattern much like that seen in vivo in postpubertal mammary glands (Daniel and Silberstein, 1987). This type of pattern has been observed by Berdichevsky et al. (1994) when the human mammary cell line HB-2 is maintained in collagen type I gels.

HMECs assemble hemidesmosomes in vivo (Watson et al., 1988). In vitro they are also capable of forming hemidesmosomes, although this generally takes up to 14 days following plating on tissue culture substrates (Bergstraesser et al., 1995). Like HMECs in vivo, MCF-10A cells express the major components of hemidesmosomes as determined by immunoflu-

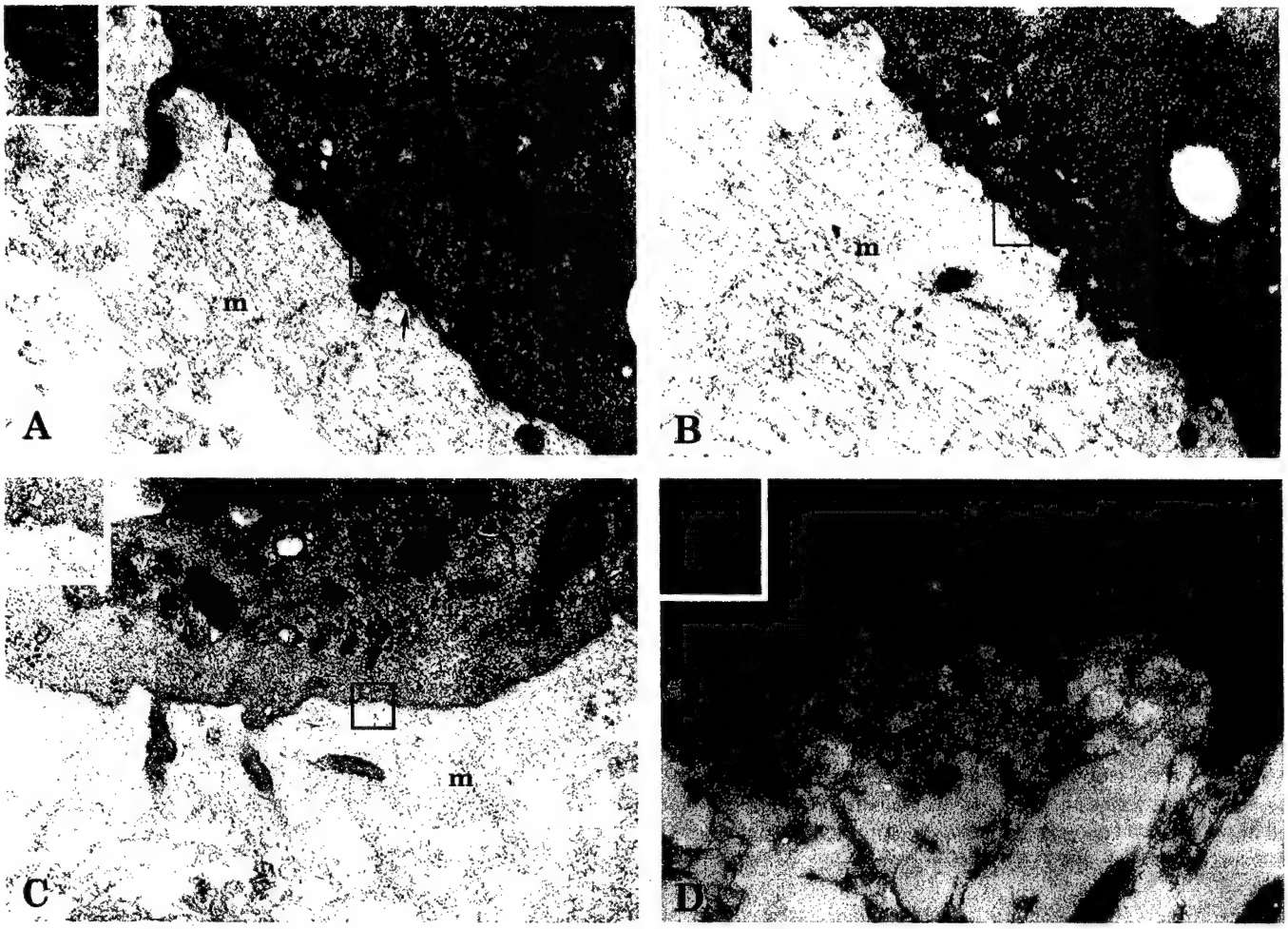

Fig. 8. MCF-10A cells assemble hemidesmosomes on matrigel but this is inhibited by integrin and laminin-5 antibodies. MCF-10A cells were plated onto matrigel in the presence of control IgG (A), or antibodies which block the function of α6 integrin (GoH3, B), α3 integrin (P1B5, C), or laminin-5 (C2-9, D). After 24 hours the cells on matrigel were fixed for electron microscopy. Note that in A there are three hemidesmosome-like structures along the region of cell-matrigel interaction (arrows). One of these (in the box) is shown at higher power in the inset. It possesses an electron dense cytoplasmic plaque and also a sub-basal dense plate. There are no obvious hemidesmosomes in cells in contact with matrigel in B-D (higher power views of these regions are shown in the insets). m, matrigel. Bars: (A), 500 nm; (inset), 60 nm.

orescence, immunoblotting and immunoprecipitation. Moreover, MCF-10A cells readily assemble hemidesmosome-like structures within 24 hours after plating onto uncoated glass coverslips i.e. much faster than their normal counterparts. The speed of hemidesmosome appearance in MCF-10A cells was the more surprising since earlier work had suggested that MCF-10A cells were unable to assemble bona fide hemidesmosomes in vitro (Tait et al., 1990).

When maintained on matrigel, MCF-10A cells assemble hemidesmosomes at sites of cell-matrigel interaction. Consistent with this, a hemidesmosome associated matrix component and its receptor, namely laminin-5 and α6β4 integrin, are distributed at sites of MCF-10A cell-matrigel interaction. Such observations triggered our interest in the potential role of hemidesmosome components in branching morphogenesis of MCF-10A cells. Since it is already established that laminin-5 and α6β4 integrin heterodimer are essential for hemidesmosome assembly, we have been able to assay the role of hemidesmosomes in branching morphogenesis of MCF-10A cells by using antibodies which inhibit both the activities of laminin-5 and α6β4 integrin (Jones et al., 1991; Kurpakus et al., 1991; Spinardi et al., 1995; van der Neut et al., 1996; Georges-Labouesse et al., 1996; Baker et al., 1996).

Function blocking antibodies directed against laminin-5 not only prevent hemidesmosome assembly in MCF-10A cells maintained on matrigel but also significantly inhibit branching morphogenesis. Similarly, antibody GoH3, which blocks α6 integrin function, inhibits both hemidesmosome formation and MCF-10A morphogenesis. Since the α6 integrin subunit is known to preferentially bind β4 integrin in cells which coexpress both of its β1 and β4 integrin partners, as is the case in MCF-10A cells, the inhibitory effects of GoH3 antibodies on MCF-10A cells likely impact the function of the hemidesmosome-associated α6β4 integrin heterodimer (Giancotti et al., 1992; S. E. Baker and J. C. R. Jones, unpublished observations).

Indeed, we assume that matrigel, or more specifically its laminin-1 component, provides an initial framework for MCF-10A attachment and triggers a series of morphogenetic events (Streuli et al., 1995). This includes secretion of laminin-5

which then induces the MCF-10A cells to nucleate the assembly of their own hemidesmosomes, a process requiring laminin-5/α6β4 integrin interaction. We suggest that the formation of the latter complex is necessary to complete branching morphogenesis.

The idea that hemidesmosomes may be involved in morphogenetic events is supported indirectly by recent reports which indicate that hemidesmosomes are sites of signal transduction (Maniero et al., 1995, 1996). For example, the β4 subunit of the α6β4 hemidesmosome associated integrin possesses an unusually long cytoplasmic tail which is associated with one or more protein kinases (Tamura et al., 1990; Maniero et al., 1995). The latter are believed to be involved in a matrix induced cascade of phosphorylation events resulting in phosphorylation not only of the β4 integrin subunit but also of a recently identified protein of 80 kDa (Xia et al., 1996; Maniero et al., 1995).

Laminin-5 and α6 antibodies are not exclusive in their abilities to block morphogenesis of MCF-10A cells in matrigel. A function perturbing α3 integrin antibody, P1B5, is also capable of inhibiting matrigel induced branching morphogenesis of MCF-10A cells. The α3β1 integrin heterodimer is not a component of the hemidesmosome but, like α6β4 integrin is a receptor for laminin-5 (Carter et al., 1990, 1991). In in vitro assays, it has been shown that cell interaction with laminin-5 is initiated by the α3β1 integrin heterodimer (Carter et al., 1991). Subsequently laminin-5 appears to 'switch' receptors and binds to the α6β4 integrin as a prelude to hemidesmosome assembly (Carter et al., 1990, 1991; Spinardi et al., 1995; Xia et al., 1996). Thus one explanation for the morphogenetic impact of the α3 integrin blocking antibody is that P1B5 inhibits the interaction of cells with their own laminin-5. However, we cannot discount that α3 integrin is involved in cell binding to the laminin-1 component of matrigel (Streuli et al., 1995). Of course, P1B5 may inhibit both laminin-1 and laminin-5 interactions of the MCF-10A cells.

In summary, we have identified a model system and a continuous cell line, MCF-10A, for the study of the role of hemidesmosome matrix and integrin components in tissue morphogenesis. In this model, matrigel provides a three-dimensional environment which triggers a series of cellular morphogenetic events, involving the assembly of hemidesmosomes and expression of hemidesmosome matrix and integrin components, in MCF-10A cells. Indeed, it is becoming clear that the hemidesmosome is not simply a spot weld to tether cells to connective tissue but, through the functional properties of its components, the hemidesmosome can have a profound impact on the differentiation and organization of epithelia at the tissue level.

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Cell Motility and the Cytoskeleton 37:271–286 (1997)

Laminin-5 and Modulation of Keratin Cytoskeleton Arrangement in FG Pancreatic Carcinoma Cells: Involvement of IFAP300 and Evidence That Laminin-5/Cell Interactions Correlate With a Dephosphorylation of α6A Integrin

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Under normal culture conditions, epithelial cells of the FG line, derived from a pancreatic tumor, characteristically grow in mounds and fail to flatten efficiently onto their substrate. In such cells, keratin intermediate filaments (IFs) are concentrated in the perinuclear region. Furthermore, the IF associated protein, IFAP300, primarily localizes along these keratin bundles. Additionally, α6β4 integrin heterodimers localize in streaks or spots towards the edges of cells while α3β1 integrin is predominantly at cell-cell surfaces. Neither show any obvious interaction with IF. Remarkably, upon plating FG cells into medium containing soluble rat laminin-5, FG cells rapidly adhere and spread onto their substrate. Moreover, FG cells "capture" rat laminin-5 and place it basally in circles or arcs at areas of cell-substrate interaction. Double label immunofluorescence microscopy reveals colocalization of IFAP300 as well as α6β4 and α3β1 integrin with the polarized laminin-5. Concomitantly, α6 integrin undergoes dephosphorylation on serine residue 1041. Laminin-5-induced rapid adhesion can be blocked by antibodies against the α3 integrin subunit. In contrast, while α6 integrin antibodies do not block laminin-5-induced rapid adhesion, they prevent FG cells from assuming an epithelial-like morphology. Keratin IF bundles associate with IFAP300-α6β4/α3β1 integrin complexes along the cell-substratum-attached surface of FG cells coincubated in laminin-5-containing medium. Coprecipitation results suggest that in these complexes, IFAP300 may associate with the α6β4 integrin heterodimer. Based on our results and published evidence that IFAP300 binds keratin in vitro [Skalli et al., 1994; *J. Cell Biol.* 125:159–170], we propose that laminin-5/FG cell interaction results in a novel integrin dephosphorylation event, which subsequently induces IFAP300 association with α6β4 integrin. IFAP300 then mediates the interaction of IFs with the cell surface via the α6β4 integrin heterodimer. Cell Motil. Cytoskeleton 37:271–286, 1997.

Key words: intermediate filaments; extracellular matrix; cell surface receptors

INTRODUCTION

Interactions between matrix and cells have a profound impact on cell physiology, motility and differentiation. One obvious manifestation of matrix influence is in determining cell morphology. This is primarily the result of modulation in cytoskeleton organization, induced by signals, originating in the matrix and relayed by integrins, transmembrane heterodimer matrix receptors [Hynes, 1992].

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The molecular basis by which the microfilament system of the cytoskeleton interacts with integrins and is thereby linked to the extracellular matrix has been well studied [Simon and Burridge, 1994]. In contrast, molecular interactions between intermediate filaments (IF) and the cell surface have only recently begun to be elucidated [Jones and Green, 1991; Jones et al., 1994; Kouklis et al., 1994; Skalli et al., 1994; Green and Jones, 1996]. For example, along the substratum-attached surfaces of certain epithelial cell types, IF are associated with the integrin heterodimer, α6β4, in a complex structure called the hemidesmosome [Stepp et al., 1991; Jones et al., 1994; Green and Jones, 1996]. Since the β4 integrin subunit possesses a long cytoplasmic tail of over 1,000 amino acids, it has been hypothesized that it may directly bind IF [Quaranta and Jones, 1991]. However, there are other hemidesmosomal proteins which may link IF to the α6β4 integrin complex. One of these is the 230-kD bullous pemphigoid antigen which resides in the hemidesmosome plaque close to the region where IF appear to bind. Evidence for the role of BP230 in IF-cell surface interaction is indirect and comes from analyses of mice in which BP230 has been knocked out [Guo et al., 1995]. In such mice, epidermal hemidesmosomes appear to lack IF bundle connections [Guo et al., 1995]. Another candidate for an IF-hemidesmosomal plaque linker is IFAP300, which binds keratin in in vitro assays and which, like BP230, localizes to sites of IF-hemidesmosomal plaque interaction [Skalli et al., 1994]. IFAP300 is likely related to the protein, HD1, which has also been reported to be involved in IF-hemidesmosome interactions [Hieda et al., 1992; Gache et al., 1996; Smith et al., 1996; McLean et al., 1996].

There is accumulating evidence that laminin-5 is the extracellular ligand for α6β4 integrin in the hemidesmosome and therefore is one link in the chain of molecules which integrate the keratin IF cytoskeleton with the extracellular matrix [Carter et al., 1990b; Marchisio et al., 1993; Langhofer et al., 1993; Rousselle et al., 1991; Jones et al., 1994; Burgeson et al., 1994; Spinardi et al., 1995]. The work of Langhofer et al. [1993] has provided an indication of the importance of laminin-5 in inducing IF cell surface association. These workers showed that maintenance of keratinocytes on a rat laminin-5-rich matrix led to the establishment of IF interaction with the substratum-attached surface and induced hemidesmosome assembly. Remarkably, we show here that when cells of the pancreatic carcinoma cell line, FG, are plated into medium containing soluble rat laminin-5, they adhere rapidly, placing the medium-derived laminin-5 along their substratum attached surfaces. We have chosen FG cells for our analyses because they possess the hemidesmosome-associated integrin α6β4 but lack other membrane and plaque components of the hemidesmosome including BP180 and BP230 [Jones et al., 1994; Hopkinson and Jones, 1994; Hopkinson et al., 1995]. Surprisingly, despite recent data suggesting that BP230 is necessary for IF-hemidesmosome interaction [Guo et al., 1995], when FG cells are plated into laminin-5-containing medium, IF are found along the substratum-attached surface and are associated spatially with a complex that contains laminin-5, α6β4 and α3β1 integrin as well as an IF-associated protein termed IFAP300.

MATERIALS AND METHODS

Cell Culture

804G cells were maintained in Dulbecco's Modified Eagles' Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum, penicillin (100 I.U./ml) and streptomycin (100 mg/ml). Conditioned medium was obtained from 804G cells 1 day postconfluency. FG cells were plated directly into 804G-conditioned medium, or DMEM with 10% fetal calf serum, penicillin (100 I.U./ml) and streptomycin (100 mg/ml).

Antibodies

Monoclonal antibody GoH3 against the α6 integrin subunit was purchased from ImmunoTech (Westbrook, ME). Monoclonal antibodies 3E1 (β4), P1B5(α3), P4C10 (β1) were purchased from Gibco/BRL (Gaithersburg, MD). The β4 polyclonal antibody (6945) and polyclonal antibody 6844, against the cytoplasmic domain of the integrin subunit α6A, were gifts from Dr. Vito Quaranta [Tamura et al., 1990]. Dr. Richard Hynes kindly provided the β1 integrin subunit polyclonal serum. Monoclonal antibody 5C5 against the α chain of laminin-5 have been described previously [Langhofer et al., 1993; Baker et al., 1996]. The generation of the IFAP 300 monoclonal and polyclonal antibodies was detailed in Yang et al. [1985]. A monoclonal pan-keratin antibody was purchased from Sigma Chemical Co. The mouse IgG1 monoclonal antibody 4E9G8 against α6 integrin subunit was purchased from ImmunoTech.

Immunofluorescence

Cells grown on coverslips were processed for immunofluorescence as described in Klatte et al. [1989] and Riddelle et al. [1991]. Appropriate secondary antibodies were purchased from Southern Biotechnology (Birmingham, AL). For mouse/rat double labels, goat anti-mouse fluorescein antibodies preadsorbed against rat IgG and goat anti-rat rhodamine antibodies preadsorbed against mouse IgG were purchased from Southern Biotechnology. Coverslips were viewed on an LSM10 confocal microscope (Carl Zeiss, Thornwood, NY). Unless stated, all images were taken within 0.5 μm of the substrate-

attached surface of the cell. No adjustment of focus was made when changing lasers to analyze double labels. Images were stored on Sony magneto-optical disks (Inmac, Irving, TX) and printed on a Tek Phaser IISDX color printer (Tektronics, Beaverton, OR).

Adhesion and Antibody Blocking Assays

Cells were suspended in normal medium, conditioned medium, or conditioned medium in the presence of integrin antibodies for 15 min post-trypsinization at 37°C. 5×10^4 cells were then plated into each well of a nontissue culture-treated 96-well plate (Sarsedt, Inc., Newton, NC) and incubated for 45 min at 37°C. Cells were washed extensively in Dulbecco's phosphate-buffered saline (PBS), fixed for 15 min in 3.7% formaldehyde in PBS and then incubated at room temperature with 0.5% crystal violet for 10 min. The dye was then solubilized with 1% SDS (100 µl/well) and absorbance at 595 nm was measured on a Vmax plate reader (Molecular Devices, Palo Alto, CA).

To assess the impact of antibodies on FG cell spreading, cell outlines were digitized following capture by a EDC1000 CCD camera (Electrim, Princeton, NJ). Surface areas were determined using software developed by Dr. Guenter Albrecht-Buehler at Northwestern University Medical School. Statistical analyses were performed using Microsoft Excel. Controls for these studies included the use of irrelevant mouse and rat IgGs.

Immunoprecipitation

Cells were solubilized in tris-buffered saline (TBS, pH 7.4) containing 0.5% NP-40, 2 mM CaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM leupeptin, 1 mM pepstatin and 1 µg/ml aprotinin. After clarifying at 15,000 rpm for 15 min at 4°C, the supernatant was collected and precleared with protein-G agarose beads (GIBCO). The supernatant was then rotated at 4°C with either β4 (3E1), β1 (P4C10) or IFAP300 (417D1) mouse monoclonal antibodies for 2 hr and then protein-G agarose beads were added for an additional 2 hr. The samples were centrifuged, the supernatant discarded, and the beads washed three times in a wash buffer (TBS containing 0.5% NP-40). After the final wash, the beads were boiled in Laemmli sample buffer [Laemmli, 1974] containing 10% β-mercaptoethanol (βME) for 5 min.

For ^{32}P radiolabeling, subconfluent cultures of FG cells were washed and then incubated in phosphate free medium for 30 min at 37°C. Next, cells were passaged and replated into 60-mm dishes in the presence or absence of laminin-5 in phosphate free medium. Immediately after plating, 0.1 mCi/ml of [^{32}P] orthophosphate was added to each dish. After radiolabeling, cells were rinsed in TBS and subjected to immunoprecipitation as detailed above using GoH3 antibody. To inhibit phosphatase and kinase activity, 10 mM sodium fluoride, 4 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 4 mM EDTA were added to the immunoprecipitation lysis and wash buffers. Samples were analyzed by SDS-PAGE on 12% gels. Gels were either dried and exposed to film or separated proteins were transferred to nitrocellulose which was then processed for immunoblotting with 6844 α6A polyclonal antibodies. Autoradiographs and immunoblots were scanned and quantitated using Intelligent Quantifier (Bio Image, Ann Arbor, MI).

Peptide Preparation

The peptide, NH_2-CIHAQPSDKERLTSDA-COOH, and phosphopeptide, NH_2-CIHAQP-pS-DKERLTSDA-COOH, were prepared commercially (Research Genetics, Huntsville, AL). These peptides match the amino acid sequence (residues 1036 to 1050) of a portion of the cytoplasmic domain of the α6A integrin subunit [Tamura et al., 1990]. The amino terminal cysteine residue was included for future conjugation purposes. The phosphorylated serine in the second peptide corresponds to serine residue 1041 which has been shown to be phosphorylated in vivo [Hogervorst et al., 1993]. Peptides were spotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA) which was subsequently processed for immunoblotting as detailed below.

SDS-PAGE and Immunoblotting

Whole cell extracts were made by solubilization of a confluent 100-mm dish of cells in 1 ml of Laemmli type sample buffer containing 10% βME [Laemmli, 1974]. SDS-PAGE and immunoblotting were carried out as described previously [Zackroff et al., 1984; Klatte et al., 1989]. Laminin-5 was purified from 804G-conditioned medium as previously detailed [Baker et al., 1996]. Approximately 1.0 µg bovine serum albumin (BSA) was added to 5.0 µg of laminin-5 as a protein stabilizer.

RESULTS

Impact of Laminin-5-Containing Medium on the Localization of Integrin Heterodimers α6β4 and α3β1 in FG Cells

When FG cells are plated on glass coverslips in their normal culture medium, they fail to attach by 4 hr, and at 24 hr they group together in stratified mounds as visualized by phase contrast microscopy (Fig. 1A). In contrast, these same cells adhere rapidly within less than 45 min after plating into medium conditioned by 804G cells, a rat bladder epithelial cell line which secretes laminin-5 (see also adhesion assay below) [Hormia et al., 1995; Fig. 1B]. At 24 hr following plating into 804G-conditioned medium, FG cells appear flattened and are associated together in sheets (Fig. 1C). Laminin-5 is a

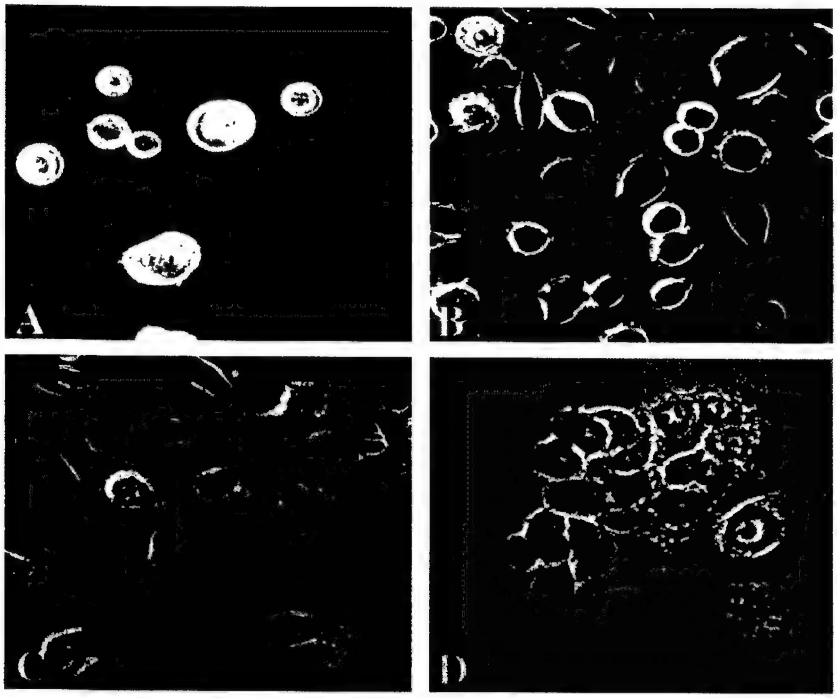

Fig. 1. FG cells were plated onto glass coverslips in control medium (**A**), 804G-conditioned medium for 1 hr and 24 hr (**B,C**), or medium supplemented with 20 μg/ml of immunopurified laminin-5 for 24 hr (**D**). Cells were viewed by phase contrast microscopy. Cells plated into normal medium (A) cluster in mounds and are poorly adherent, whereas cells plated into 804G-conditioned medium rapidly adhere and spread onto their substrate (B). At 24 hr, FG cells plated into 804G-conditioned medium (C) and medium supplemented with 20 μg/ml purified laminin-5 (D) are well spread, possessing an epithelial-like morphology. Bar = 25 μm.

good candidate for the "active" factor in the medium. To confirm this, we added purified laminin-5 at 20 μg/ml to FG cell growth medium. FG cells in this medium behave identically to cells plated into 804G-conditioned medium. For example, FG cells, at 24 hr after plating into purified laminin-5-containing medium, are well adhered to their substrate and have assembled into a squamous sheet (compare Fig. 1D with 1C). It should also be noted that FG cells are not induced to migrate under these conditions (Baker and Jones, unpublished observations).

FG cells plated into 804G-conditioned medium utilize soluble rat laminin-5 placing it in circles or arcs at the basal aspect of the cell as determined by confocal immunofluorescence microscopy using monoclonal antibodies specific for the α chain of laminin-5 (Fig. 2A [Baker et al., 1996]. These antibodies do not recognize human laminin-5 and thus, any staining observed is due to "capture" of 804G laminin-5 [Langhofer et al., 1993; Jones et al., 1994]. An identical phenomenon is observed if FG cells are plated into FG growth medium supplemented with purified laminin-5 (Fig. 2C).

We next investigated whether "capture" of laminin-5 involves cytoskeletal elements and modulation in cytoskeleton organization. We first determined potential integrin involvement. There are two likely cell surface receptors for laminin-5, namely α6β4 and α3β1 integrins [Carter et al., 1990a; Langhofer et al., 1993; Marchisio et al., 1993; Jones et al., 1994, Spinardi et al., 1995]. It should be noted that in FG cells, the α6 integrin subunit preferentially associates with the β4 integrin subunit rather than the β1 integrin subunit, consistent with the results of others (result not shown) [Giancotti et al., 1992].

FG cells were plated into either their normal medium or laminin-5-rich medium and, at various times following plating, the FG cells were processed for immunofluorescence microscopy using antibodies directed against either the α3 or the α6 integrin subunit. In normal medium at 1 hr following plating, FG cells have not adhered to their substrate. Thus, it was necessary to wait 24 hr following plating of FG cells into normal medium to evaluate integrin localization. At 24 hr, α3 integrin localizes primarily to cell-cell contacts while integrin α6 is located in areas of cell-substrate contact in a patchy pattern (Fig. 3). The rapid attachment of FG cells in 804G-conditioned medium enabled us to determine the location of α3β1 and α6β4 integrin heterodimers even at 1 hr following plating. At this time point, α6 integrin colocalizes with the α chain of laminin-5 (Fig. 4A,B). Even after 24 hr, when FG cells have grouped into sheets, there is concentration of α6 integrin at sites rich in rat laminin-5 (Fig. 4D,E). Additionally, α6 and α3 integrin subunits, both at 1 and 24 hr, codistribute in circles or arcs at the basal edges of the cells (Fig. 4G,H; only the result for the 1 hr cells is shown).

Laminin-5 and the Phosphorylation State of α6 Integrin

We next investigated the impact of culturing FG cells in the presence of laminin-5 on the physiological state of α6 integrin. For these studies we made use of monoclonal antibody 4E9G8 which has been reported to

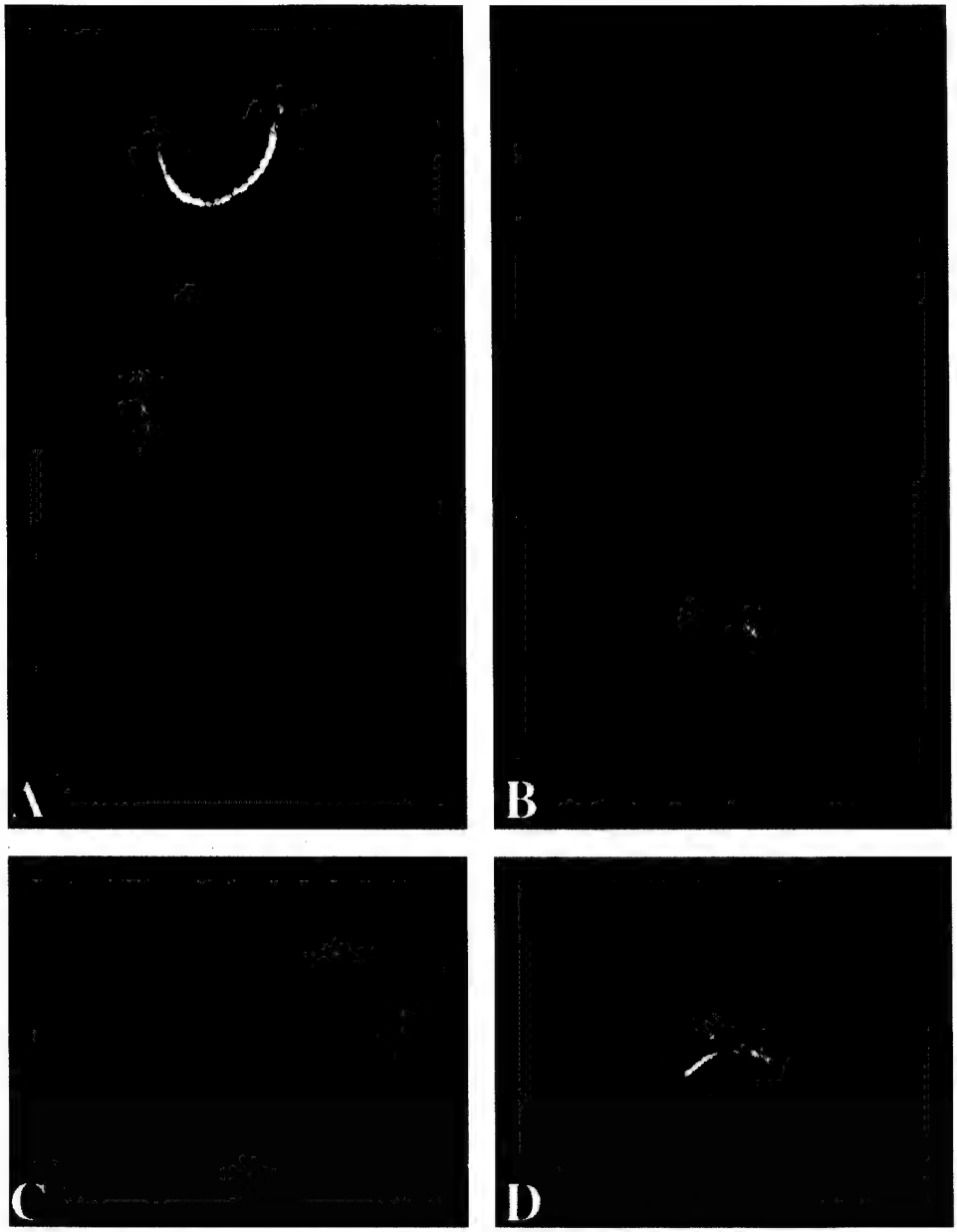

Fig. 2. FG cells were grown for 24 hr on glass coverslips in 804G-conditioned medium (**A,B**) or control medium to which 20 μg/ml of immunopurified laminin-5 was added (**C,D**). Coverslips were then processed for confocal immunofluorescence microscopy using a monoclonal antibody against the laminin-5 α chain (A,C). The staining patterns generated on cells grown in conditioned medium (A) and control medium supplemented with immunopurified laminin-5 (C) are similar; cells have "captured" the soluble laminin-5 and concentrated it, basally, in circles or arcs. The plane of focus is at the base of the cell. B and D, phase contrast images. Bar = 25 μm.

be specific for a phosphorylation-sensitive epitope on α6A integrin subunit, the predominating splice variant expressed in FG cells [Quaranta et al., 1994; Hogervorst et al., 1993]. The antibody fails to recognize FG cells under their normal culture conditions as determined both by immunofluorescence and immunoblotting (Figs. 5A, 6A). Conversely, in FG cells incubated in laminin-5-containing medium, 4E9G8 antibody shows an arc or circle of staining towards the cell-substratum interface (Fig. 6C). Moreover, 4E9G8 antibodies localize with a subpopulation of α6 integrin as shown in double label studies using GoH3 antibodies (Figs. 6E,F). In addition, 4E9G8 antibody reacts with the α6A "light" chain, migrating at 25 kD, in extracts of FG coincubated in laminin-5-containing medium (Fig. 5A).

To further define the epitope of 4E9G8 antibody, we generated peptides consisting of residues 1036–1050 of the α6A integrin subunit cytoplasmic domain. In one of

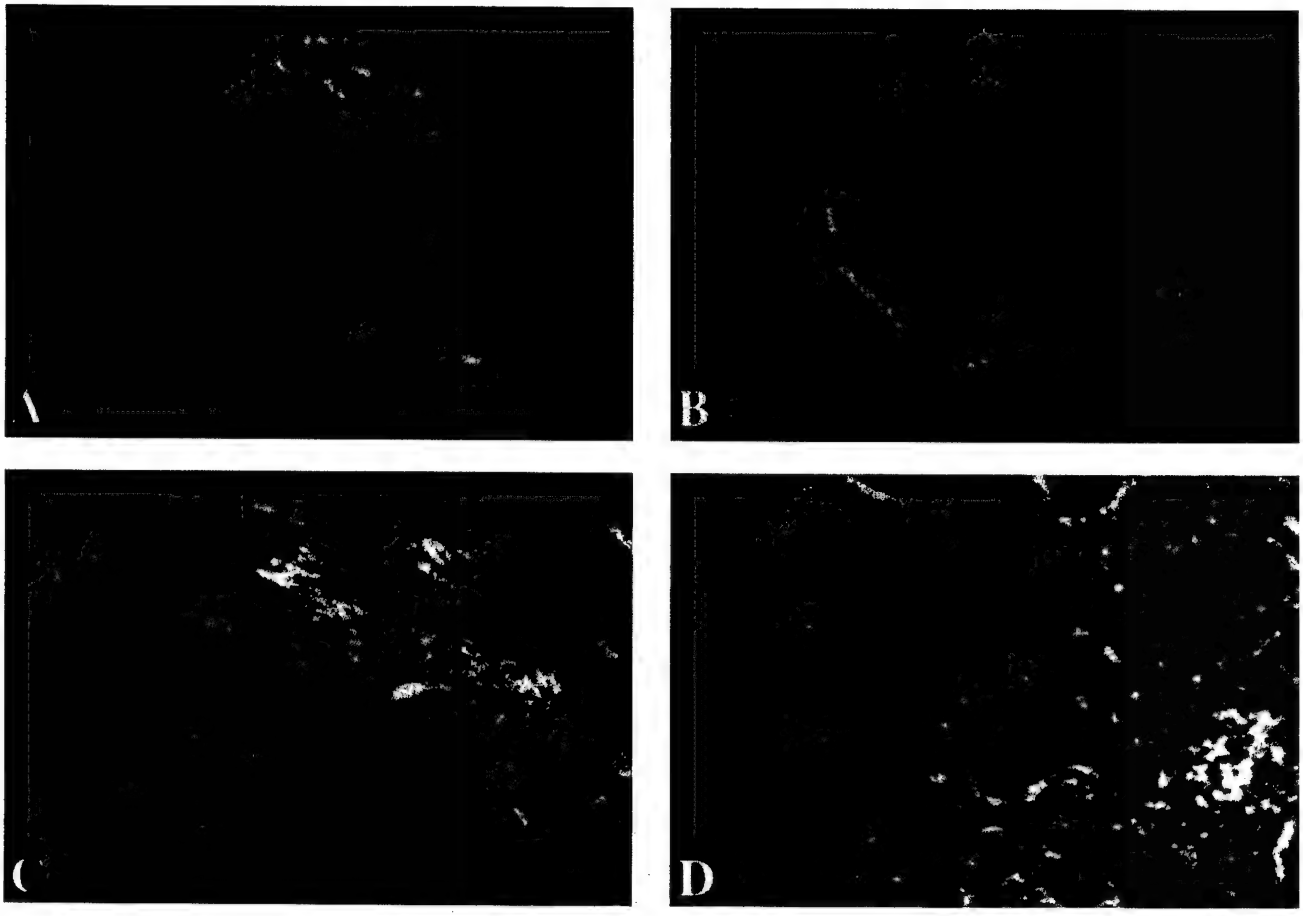

Fig. 3. FG cells were plated into control medium and 24 hr later processed for immunofluorescence using monoclonal antibodies to α3 (**A**) and α6 (**C**) integrin subunits. Confocal microscopy shows that α3 staining is concentrated at cell-cell borders (A) while α6 staining consists of patches and streaks in a plane of focus located at the basal aspect of the cell. **B** and **D**, phase contrast images. Bar = 25 μm.

our peptides, serine residue 1041 was phosphorylated. This peptide was not recognized by 4E9G8 antibodies unlike its nonphosphorylated counterpart (Fig. 5B). Both phosphorylated and nonphosphorylated peptides are recognized by a rabbit serum 6844 directed against the α6A integrin cytoplasmic domain (Fig. 5B).

To gain independent confirmation that the α6 integrin undergoes a dephosphorylation in FG cells maintained in the presence of laminin-5, we undertook an in vivo phosphorylation assay (Fig. 5C). The α6 integrin was immunoprecipitated using GoH3 antibodies from ^{32}P-radiolabeled FG cells, maintained either in the absence or presence of laminin-5. Equal amounts of α6 integrin, precipitated from FG cells maintained under the two distinct conditions, were loaded onto gels as shown by the immunoblot using serum 6844. There is an apparent 37% decrease in the level of phosphorylation of the 25-kD α6 integrin light chain in cells maintained in the presence of laminin-5 when compared with control cell immunoprecipitates (Fig. 5C).

α3β1 Integrin is Involved in Rapid Attachment While α6β4 Integrin is Involved in Development of Epithelial Morphology in FG Cells Incubated in Laminin-5-Containing Medium

Is the rapid adherence and spreading of FG cells in the presence of laminin-5-rich medium integrin-dependent? Cells were removed from their culture dishes and suspended for 15 min in their normal medium or laminin-5-rich medium with and without addition of "blocking" antibodies against α3 or α6 integrins (P1B5 at a 1:20 dilution and 50 μg/ml of GoH3, respectively) [Carter et al., 1990a; Sonnenberg et al., 1988]. Cells were then plated into microtiter wells and allowed to attach for 45 min. After extensive washing, adherent cells were fixed and stained with crystal violet.

FG cells in laminin-5-rich medium, preincubated in a 1:20 dilution of α3 blocking antibody P1B5, fail to attach to their substrate in our rapid adhesion assay (within 45 min of plating; Fig. 7). However, between 12

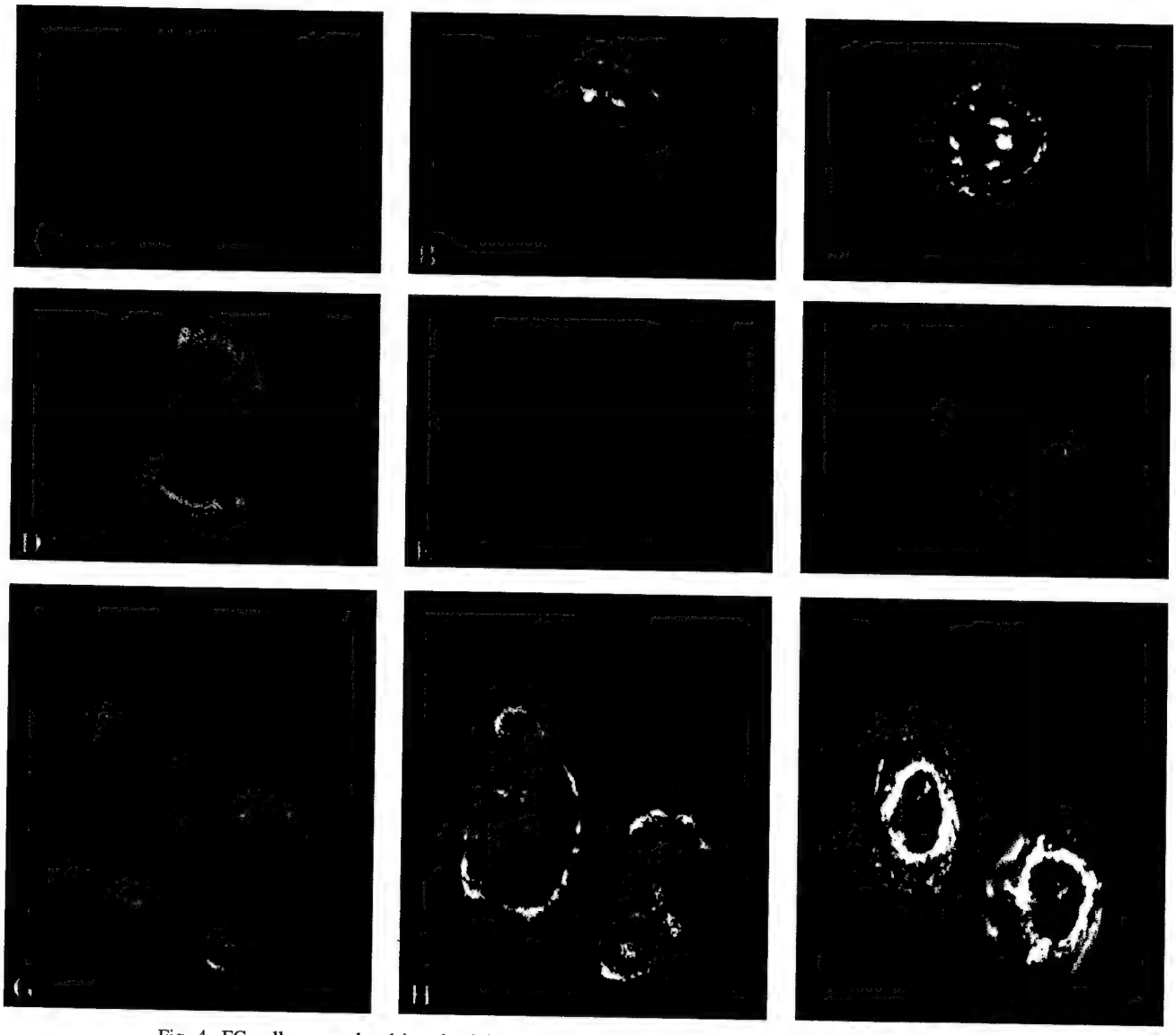

Fig. 4. FG cells were plated into laminin-5-rich medium for 1 hr (**A,B,G,H**) or 24 hr (**D,E**) and then processed for double label immunofluorescence confocal microscopy using either a combination of antibodies against α6 integrin (A,D) and laminin-5 α chain (B,E) or antibodies against α6 (G) and α3 (H) integrin subunits. Rat laminin-5 α chain antibodies (B,E) colocalize with α6 integrin (A,D) at the base of the cells although there are areas of α6 integrin distribution without associated rat laminin-5. At 1 hr, both the α6 (G) and α3 (H) subunits colocalize in circles at the base of cells. The plane of focus is at the region of cell-substrate interaction. **C,F** and **I**, phase contrast images of cells. Bars = 10 μm.

and 24 hr, the α3-blocked FG cells eventually adhere to their substrate; their attachment could be mediated by integrins other than those containing the α3 subunit although we cannot rule out the possibility that the blocking antibody has been degraded or internalized after 24 hr (Fig. 8A). At this time point, the FG cells are poorly spread as determined by phase contrast microscopy (Fig. 8A). Those FG cells incubated in 50 μg/ml of blocking α6 antibody GoH3 rapidly attach in 804G-conditioned medium, as efficiently as FG cells plated into 804G-conditioned medium (Fig. 7). However, despite their rapid adherence, the GoH3 antibody-treated cells do not exhibit a flattened fried egg appearance even at 24 hr following plating (contrast Figs. 1C and 8B). Rather, they appear spindly (Fig. 8B). Both rat and mouse irrelevant IgGs had no impact on rapid adhesion or spreading of FG cells in medium containing laminin-5 (results not shown). To quantify cell spreading, we undertook morphometric analyses of FG cells. The surface areas covered by FG cells incubated in laminin-5-containing medium and laminin-5-containing medium in the presence of 50 μg/ml control rat IgG are 429 ± 26 μm^2 (n = 39) and 542 ± 39

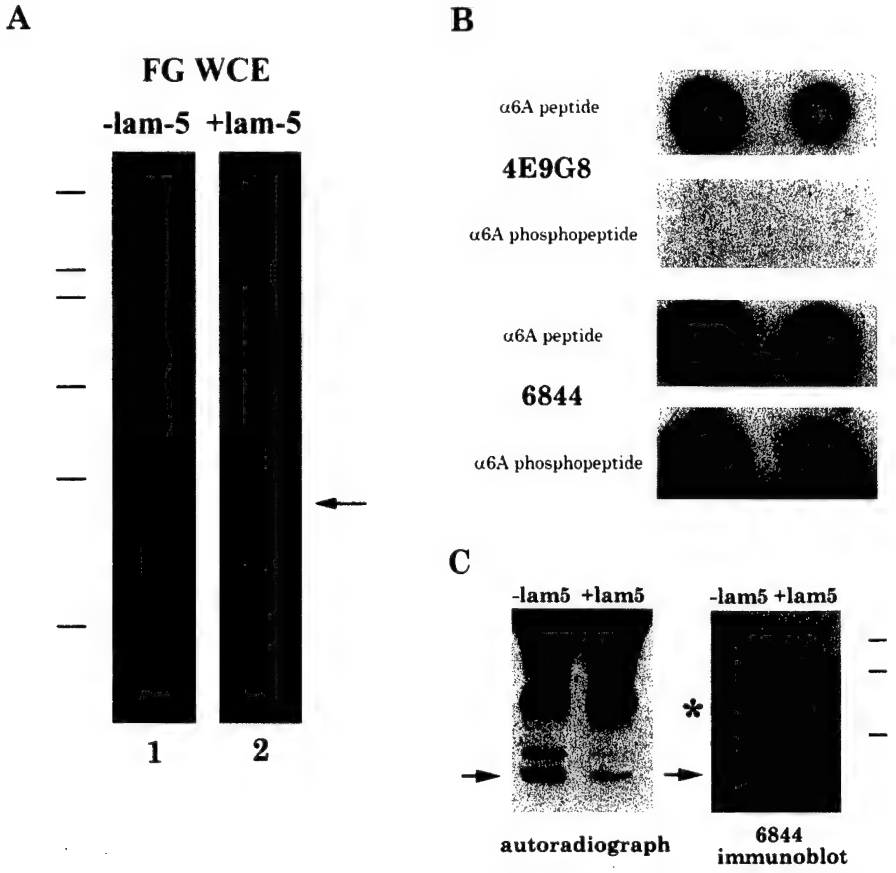

Fig. 5. **A:** Approximately 10 μg of extracts of FG cells maintained for 24 hr in their normal medium (**lane 1**) or for 6 hr in laminin-5-rich medium (**lane 2**) were prepared for SDS-PAGE on 12% gels. Separated polypeptides were then transferred to nitrocellulose and subsequently processed for immunoblotting using 4E9G8 antibody. The latter shows reactivity with a 25-kD protein only in lane 2. Molecular weight standards indicated by dashes are 200, 97, 66, 45, 31 and 21 kD. **B:** The α6A integrin cytoplasmic nonphospho- and phospho-peptides at 1 mg/ml or 0.1 mg/ml (from left to right) were spotted onto PVDF membrane. The PVDF pieces were then processed for immunoblotting either using 4E9G8 monoclonal antibody or serum 6844. Both peptides are recognized by antibodies in the 6844 serum, whereas 4E9G8 fails to recognize the phospho-peptide. **C:** FG cells, maintained in the absence (−LN5) or presence (+LN5) of rat laminin-5, were labeled with ^{32}P. The α6 integrin subunit was then immunprecipitated from the labeled cells using monoclonal antibody GoH3. Equal amounts of precipitated α6 integrin were subjected to SDS-PAGE and were either transferred to nitrocellulose and processed for immunoblotting using the α6A integrin serum, 6844, or visualized by autoradiography. The reactivity of the 6844 antibodies on the immunoblot indicates that equal amounts of α6A integrin from the FG cells have been loaded onto the two lanes. This was confirmed by scanning densitometry. In contrast, the autoradiograph shows an apparent 37% decrease in the level of phosphorylation of α6A in the GoH3 antibody precipitates derived from FG cells maintained in the presence of laminin-5 (arrow). The asterisk marks cross-reactivity of the blotting secondary antibody with the precipitated IgG. Molecular weight standards are (from top to bottom) 97, 66, and 45 kD. WCE, whole cell extract.

μm^2 (n = 46), respectively. In contrast, FG cells plated into laminin-5-containing medium in the presence of 50 μg/ml of GoH3 antibodies cover a surface of 256 ± 18 μm^2 (n = 46).

Laminin-5 Impacts the Organization of the Keratin Cytoskeleton in FG Cells

We next investigated the impact of laminin-5 on the keratin IF network of FG cells as well as on the IF-associated protein IFAP300 [Yang et al., 1985]. FG cells were prepared for immunofluorescence with an antiserum against keratin following plating in either their normal medium at 24 hr or medium conditioned by 804G cells at 1 hr. In the former case, keratin bundles are concentrated in the perinuclear region of the rounded FG cells (Fig. 9A, inset). Double label immunofluorescence reveals that IFAP300 colocalizes with the keratin IF of such cells (Fig. 9B, inset). In contrast, IFAP300 is concentrated in circles towards the cell periphery along regions of cell-substrate interaction, where it colocalizes with α6 integrin, in FG cells maintained in 804G-conditioned medium (Figs. 9D, inset; 10A,B). Remarkably, IFAP300 does not stain the lengths of the extensive cytoplasmic bundles of keratin IF as it does in control cells (Fig. 9D,E, insets). Rather, keratin IFs are contained within the circle of IFAP300 antigen (Fig. 11A,B).

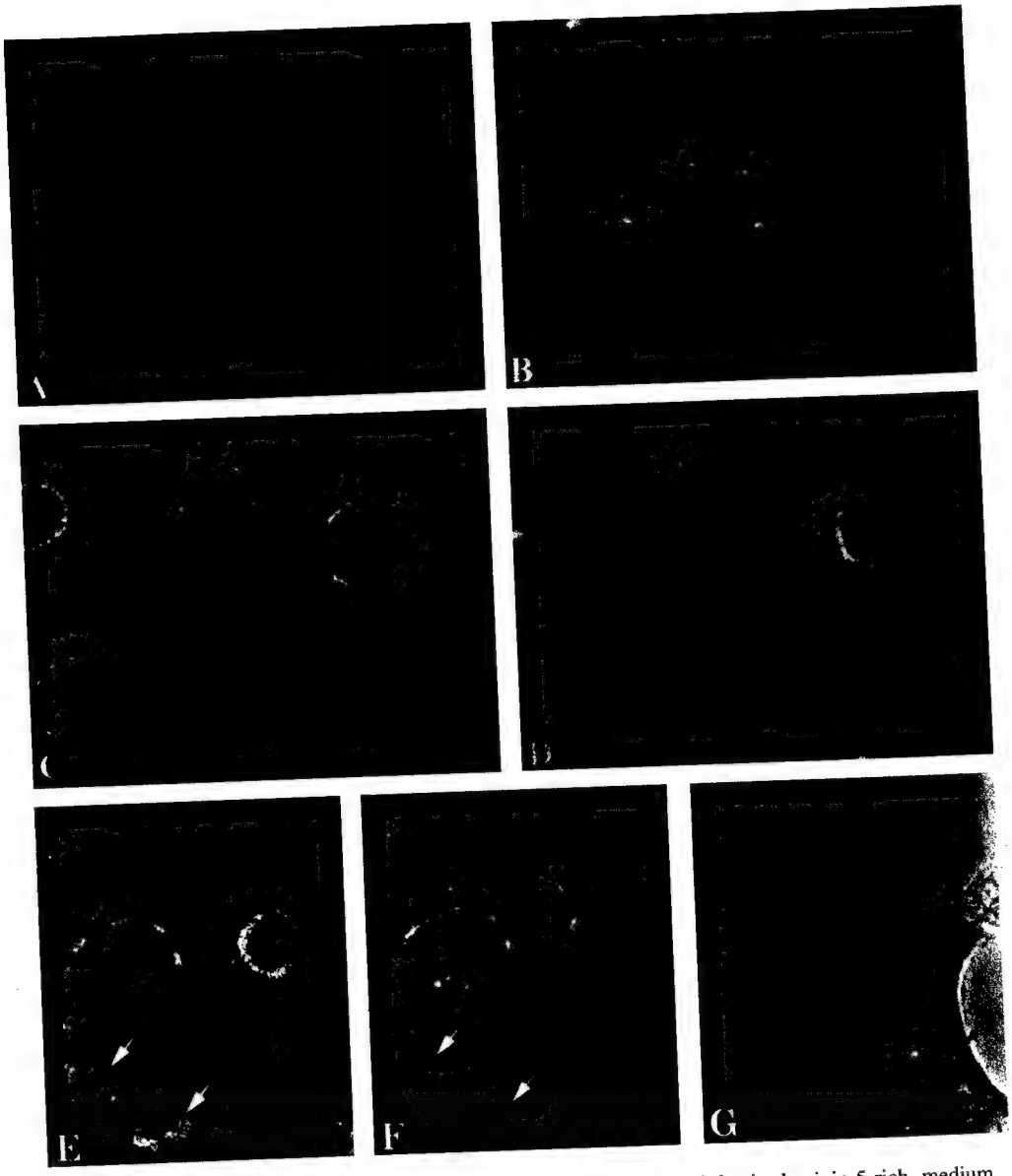

Fig. 6. At 24 hr following plating, FG cells maintained in their normal medium were processed for indirect immunofluorescence using the α6 monoclonal antibody 4E9G8 (**A**). The antibody generates no obvious staining pattern (the focal plane shown is close to the substratum attached surface of the cells), particularly when compared to FG cells stained with the α6 antibody GoH3 (Fig. 4A,G). In contrast, in FG cells maintained for 6 hr in laminin-5-rich medium, antibody 4E9G8 generates staining in circles (**C**). These circles colocalize with circles stained by GoH3 antibodies (open arrows, **E** and **F**). However, it should be noted that GoH3 staining is more extensive than that of 4E9G8 antibodies (closed arrows, E and F). **B, D** and **G** show phase contrast images of the cells. Bar in B = 10 μm; bar in G = 25 μm.

Moreover, IFs are found at areas rich in both IFAP300 and α6 integrin (Fig. 11). This is similar to the way microfilament bundles often appear to terminate at sites of vinculin-containing focal contacts, sites enriched in integrin heterodimers (Burridge et al., 1988).

IFAP 300 and α6β4 Integrin can be Coimmunoprecipitated From Cells Maintained in Laminin-5-Containing Medium

The morphological studies detailed above suggest the possibility that IFAP300 may be involved in anchorage of IF to a membrane complex containing α6β4 and α3β1 integrin heterodimers when FG cells are cultured in medium containing laminin-5. To assess potential protein-protein interactions within this complex, immunoprecipitations from FG cells cultured in their normal medium or medium containing laminin-5 were performed with IFAP300 monoclonal antibody, the β4 monoclonal antibody (3E1), or a β1 monoclonal antibody (P4C10). To analyze these immunoprecipitations, they were transferred to nitrocellulose and the resulting blots were probed either with IFAP300 monoclonal antibody or an

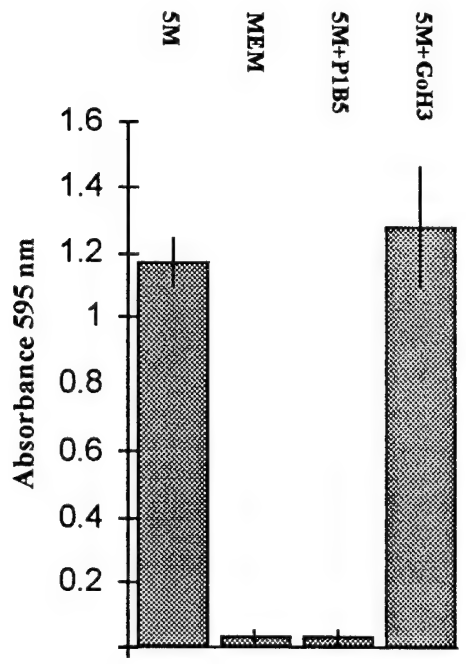

Fig. 7. FG cells were incubated for 15 min at 37°C in laminin-5-rich medium (5M), control medium (MEM), laminin-5-rich medium containing a 1:20 dilution of blocking antibody to the α3 integrin subunit (5M + P1B5) and laminin-5-rich medium containing 50 μg/ml α6 integrin subunit blocking antibody (5M + GoH3) and then plated into a 96-well plate. After a further 30 min at 37°C, the plate was washed and adherent cells were fixed and stained with crystal violet. Absorbance was measured at 595 nm. The average of readings derived from eight wells for each of the four different conditions is shown. Standard deviations are indicated by the vertical lines. Cells plated into α6 blocking antibody in laminin-5-rich medium (5M + GoH3) adhere as well as FG cells which are plated into laminin-5-rich medium alone (5M). In contrast, barely detectable numbers of FG cells attach in MEM or when α3 integrin blocked FG cells are plated in laminin-5-rich medium (5M + P1B5).

antiserum against β4 integrin (6945). A 200-kD species is recognized by the β4 integrin antiserum in 3E1 and IFAP300 antibody immunoprecipitates from FG cells cultured in laminin-5-containing medium but is not present in IFAP300 immunoprecipitates from FG cells cultured in their normal medium (Fig. 12). Conversely, a 300-kD polypeptide is recognized by IFAP300 antibodies in IFAP300 and 3E1 immunoprecipitations of FG cells maintained in laminin-5-containing medium (Fig. 12B). IFAP300 is not coprecipitated by 3E1 antibodies from extracts of FG cells cultured in their normal medium (Fig. 12A). β1 integrin antibodies do not coprecipitate detectable quantities of either β4 integrin subunit or IFAP300 protein regardless of culture conditions (Fig. 12).

DISCUSSION

We show here that FG cells plated into medium containing soluble laminin-5 are able to "capture" laminin-5, placing it in arcs or circles along regions of cell-substratum attachment. This phenomenon is not peculiar to FG cells since we have made similar observations in a variety of other human epithelial cell types. Our decision to concentrate our analyses on FG cells was primarily influenced by the dramatic impact of laminin-5-containing medium on their morphology. In the present study, we have extended the latter observations by analyzing whether integrins are involved in this "capture" process and whether the rapid attachment, spreading and morphological changes in FG cells plated into a laminin-5-supplemented medium are correlated with changes in the cytoskeleton.

Impact of Laminin-5 on the Integrins of FG Cells

Immunofluorescence observations of FG cells coincubated in laminin-5-containing medium reveal that "captured" laminin-5 colocalizes with both α3β1 and α6β4 integrin heterodimers. We are intrigued by the mechanism underlying the apparent incorporation of laminin-5 into the matrix of FG cells. One possibility is that FG cells "pull" matrix components directly from the medium and then, in some way, polarize this material. Alternatively, laminin-5 may coat glass or plastic substrate very quickly, before the cells have even begun to adhere. Following attachment of the FG cells to this surface, the cells may simply concentrate the laminin-5 into circles and arcs. These are obviously important issues which are the subject of some of our ongoing studies.

In FG cells maintained under their normal culture conditions, α3β1 integrin localizes to sites of cell-cell interaction. Laminin-5 induces a very different distribution of this integrin complex, i.e., it becomes basally polarized. Consistent with the latter, our adhesion assays provide evidence that α3β1 integrin is involved in the rapid substrate attachment of FG cells in laminin-5-containing medium. Comparable results have been presented by others who have shown that initial attachment of epidermal cells to epiligrin/kalinin (a human form of laminin-5) is mediated by α3β1 integrin [Carter et al., 1991; Rousselle and Aumailley, 1994]. On the other hand, our α6 integrin-blocked cells rapidly adhere in laminin-5-containing medium but develop a spindly morphology and are less spread than unblocked FG cells. Thus, α6β4 integrin is involved in the spreading of FG cells induced by laminin-5.

"Capture" of laminin-5 is temporally and spatially correlated with an apparent dephosphorylation of the α6A integrin subunit, the major α6 splice variant found in FG cells [Quaranta et al., 1994]. In particular, our in vivo phosphorylation analyses reveal that there is a 37% decrease in phosphorylation of the α6 integrin subunit when FG cells are cultured in laminin-5-containing medium. That not all of the α6 integrin in FG cells becomes dephosphorylated in the presence of laminin-5

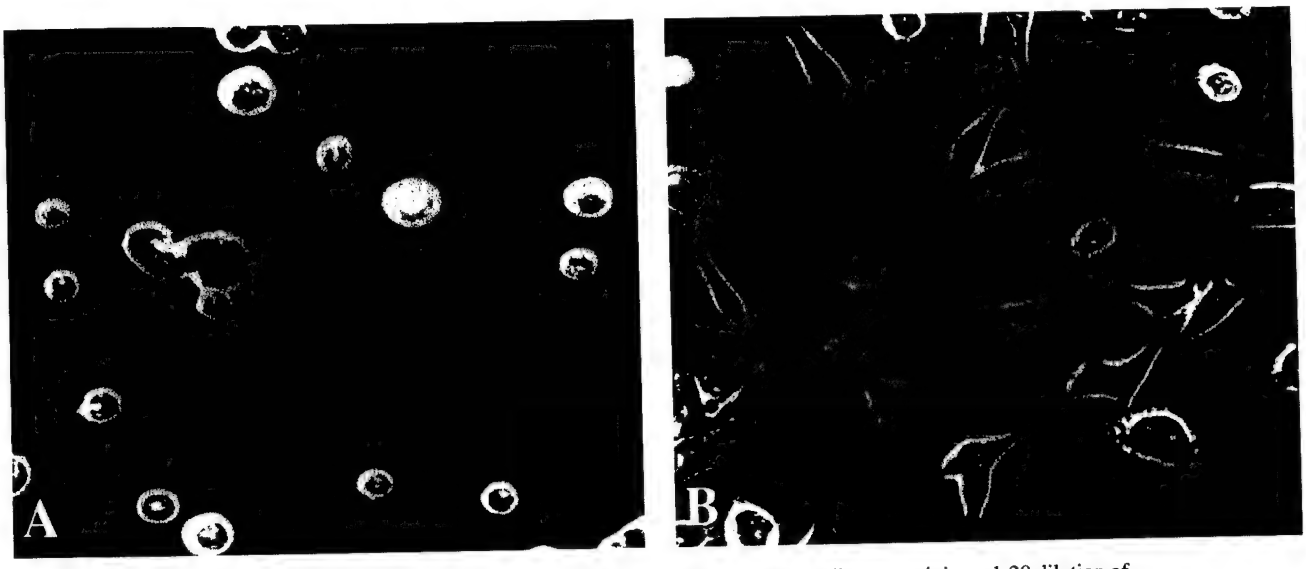

Fig. 8. FG cells were incubated for 15 min at 37°C in laminin-5-rich medium containing a 1:20 dilution of the blocking antibody to the α3 integrin subunit, P1B5 (**A**) or 50 μg/ml of the α6 integrin subunit blocking antibody, GoH3 (**B**). The treated cells were then plated in the same medium onto glass coverslips and, 24 hr later, the cells were visualized by phase contrast microscopy. Note that in A, FG cells are poorly adherent, while in B the cells are spindle-shaped. Bar = 50 μm.

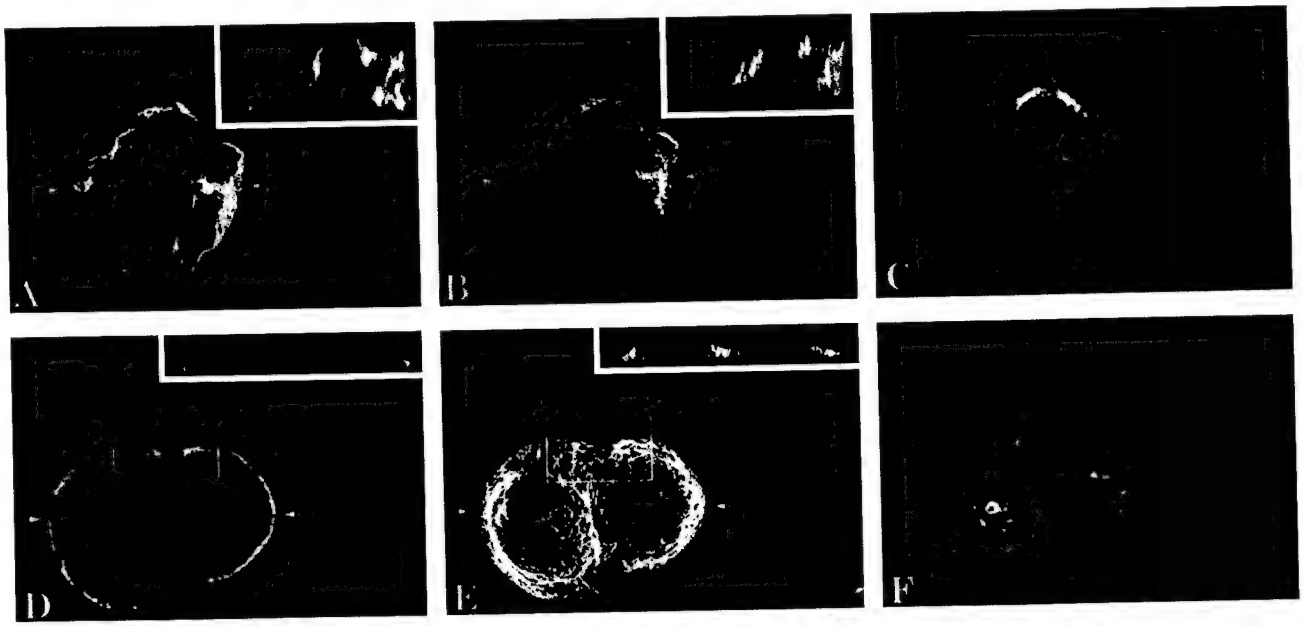

Fig. 9. FG cells maintained in their normal medium were processed 24 hr later for double label immunofluorescence using a monoclonal antibody against IFAP300 (**A**) and a keratin antiserum (**B**). Under these circumstances, IFAP300 is associated with the cytoplasmic keratin system in FG cells (**insets** in A and B show computer-generated cross-sections of cells). FG cells plated into laminin-5-rich medium for 1 hr were also processed for double label immunofluorescence using antibodies directed against IFAP300 (**D**) and keratin (**E**). In D, IFAP300 is polarized, localizing to regions of cell-substrate interaction as shown in the computer-generated cross-sectional profile (D, **inset;** compare with inset in A). Keratin filament bundles extend to the region of IFAP300 staining (E). Arrows in A,B,D and E indicate the planes of section from which the computer-generated cross-sections of cells are derived. The boxed regions in D and E are shown at high power in Figure 11A and B, respectively. **C** and **F,** phase contrast images. Bar = 25 μm.

is supported by our immunofluorescence studies (see Fig. 6). When FG cells, which have been cultured in the presence of laminin-5, are processed for double label immunofluorescence using either 5C5 and GoH3 antibodies or 4E9G8 and GoH3 antibodies, only a subpopulation of α6 integrin appears to be in a dephosphorylated state, this subpopulation showing the same localization as captured laminin-5 as well as IFAP300 (see, for example,

Fig. 10. FG cells, 1 hr following plating into laminin-5-rich medium, were processed for double label immunofluorescence using monoclonal antibodies against α6 integrin (**A**) and IFAP300 (**B**). Note that these antibodies generate similar staining patterns. **C:** phase contrast image. Bar = 10 μm.

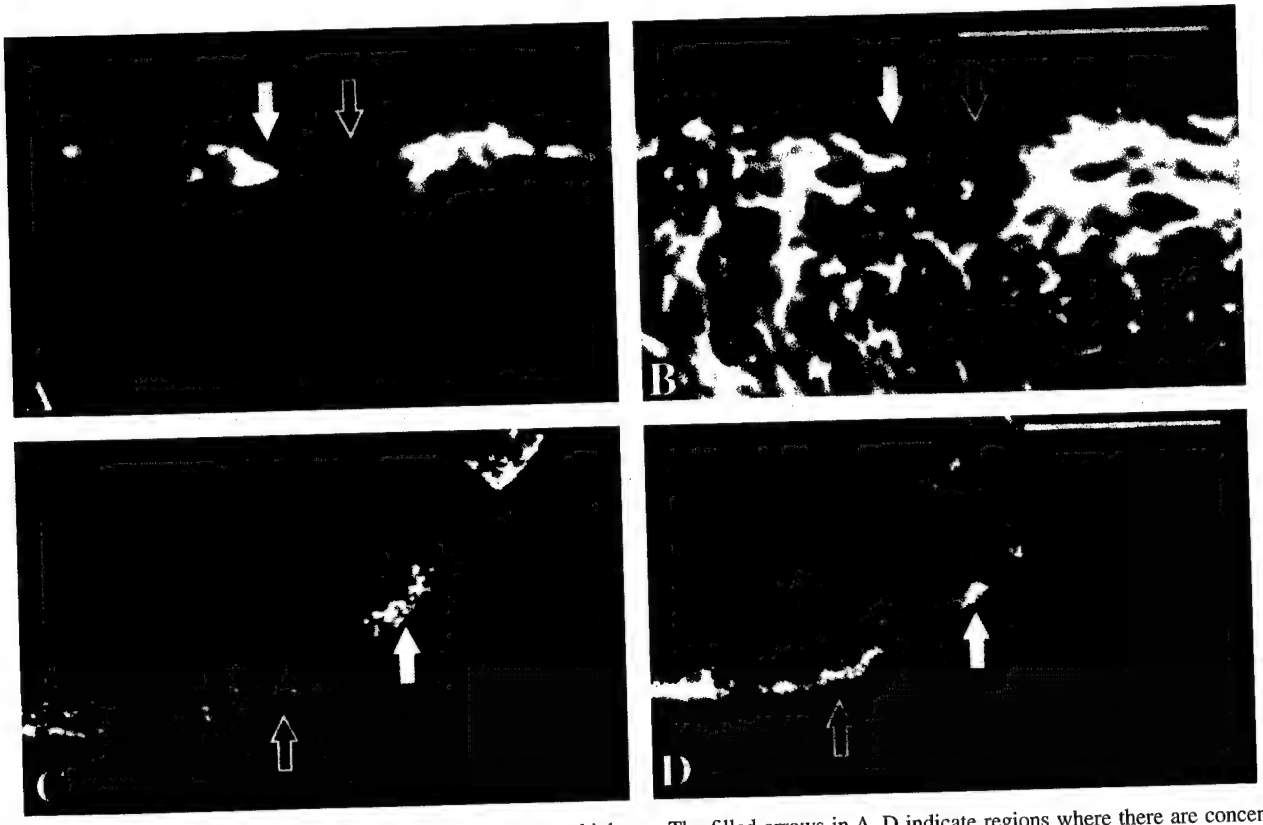

Fig. 11. Regions boxed in Figure 9D and E are shown here at higher power in **A** and **B,** respectively. In **C** and **D,** FG cells, 1 hr after plating into laminin-5-rich medium, were processed for double labels using monoclonal antibodies against α6 integrin (C) and keratin (D). The plane of focus is close to the substratum attached surfaces of the cells. The filled arrows in A–D indicate regions where there are concentrations of IFAP300 (A) or α6 integrin (C) that colocalize with keratin (B,D). The open arrows indicate where keratin bundles appear to associate IFAP300 and α6 integrin. Bars = 10 μm.

Figs. 4D,E; 6E,F; and 10). Indeed, we propose that dephosphorylation of α6 integrin is intimately involved in laminin-5-induced reorganization of the IF cytoskeleton, which is described here.

Dephosphorylation of α6A increases the affinity of α6β1 integrin for its matrix ligand laminin-1 according to Hogervorst et al. [1993]. Thus, one might speculate that initial attachment of FG cells to laminin-5 via α3β1 integrin results in activation of a phosphatase which dephosphorylates α6A. We hypothesize that this then leads to activation of the α6β4 heterodimer, increasing its affinity for laminin-5. Indeed, in support of our hypothesis, Xia et al. [1996] have reported that there is a stimulation of phosphatase activity when cells interact with laminin-5, although the exact identity of the proteins undergoing dephosphorylation was not determined by these workers. Even though we find this an attractive hypothesis, we cannot rule out the possibility that the dephosphorylation of α6A integrin induced by laminin-5 is independent of α3β1 integrin involvement.

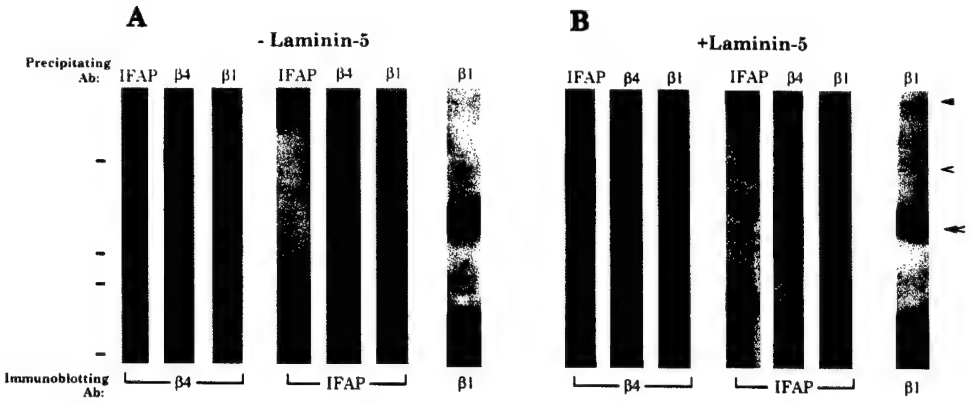

Fig. 12. FG cells were cultured in their normal medium (**A,** −Laminin-5) or in the presence of laminin-5 (**B,** +Laminin-5). Cell extracts were processed for immunoprecipitation using monoclonal antibodies against IFAP300, β4 integrin and β1 integrin (indicated at the top of each lane). Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose and then processed for immunoblotting using a polyclonal rabbit serum against the β4 integrin subunit, monoclonal antibodies against IFAP300 or a polyclonal anti-β1 integrin serum (indicated at the bottom of each lane). IFAP300, migrating close to the top of the lanes, is indicated by an arrowhead, β4 integrin is marked by an open arrow while β1 integrin is marked by a double-headed arrow. The identity of the lower molecular weight species in the IFAP300 immunoblots is unclear. There is considerable variability in these from precipitate to precipitate as is shown here and may indicate proteolysis of IFAP300 during the precipitation process. Molecular weights of 200, 116, 98 and 66 kD are indicated by short dashes to the left of the figure.

Impact of laminin-5 on the Keratin Cytoskeleton of FG Cells

We show here that laminin-5 induces the assembly of a complex that links laminin-5 indirectly to the intermediate filament cytoskeleton. We have identified IFAP300 in this complex and have shown that the β4 integrin subunit and IFAP300 are coimmunoprecipitated by antibodies to either protein. Taken together with data showing that IFAP300 interacts with keratin in vitro [Skalli et al., 1994], we speculate that keratin IF bind indirectly to the cytoplasmic domain of β4 integrin via IFAP300. Indeed, Spinardi et al. [1993] generated a mutant β4 integrin in which the 303 C-terminal most amino acids, encompassing two fibronectin type-III domains and the region between them, had been deleted. The mutant β4 protein failed to incorporate into hemidesmosome structures in 804G cells leading to speculation that this domain is responsible for hemidesmosome interaction with the cytoskeleton. To test this possibility we plan to assess whether the same domain of β4 integrin binds IFAP300 using molecular genetic techniques.

IFAP300 was originally characterized in fibroblast cells but it is also present in epithelial cells as well as cells in the nervous system [Yang et al., 1985; Skalli et al., 1994] (Goldman, unpublished observations). Likewise, α6β4 integrin expression has been demonstrated in a wide variety of epithelial tissues, endothelial cells as well as in Schwann cells [Sonnenberg et al., 1990]. Indeed, we predict that IFAP300-α6β4 complexes are involved in IF-cell surface anchorage in many cell types that do not assemble bona fide hemidesmosomes as defined by the electron microscopist. Rather, the hemidesmosome of the epidermal cell is just one example of a large family of IF associated cell-matrix attachment devices. Indeed, Uematsu et al. [1994] have already coined the term type II hemidesmosomes for an IF associated-matrix connector that possesses α6β4 integrin but lacks BP230 and any obvious membrane associated "plaque." Type II hemidesmosomes characterized by Uematsu et al. [1994] are recognized by antibodies against a high molecular weight protein component of the hemidesmosomal plaque called HD1 [Hieda et al., 1992]. There has been speculation that HD1 and IFAP300 are one and the same protein [Hieda et al., 1992; Skalli et al., 1994; Jones et al., 1994]. This possibility is supported by our unpublished observations that HD1 antibodies recognize IFAP300 immunoprecipitated from FG cells. Furthermore, HD1 shows the same pattern of localization as IFAP300 in FG cells, i.e., HD1 is IF associated under normal conditions but codistributes with β4 integrin in cells maintained in the presence of laminin-5 (Baker and Jones, unpublished observations). HD1 is now believed to be either identical to, or an isoform of, the IF associated protein termed plectin [Wiche et al., 1991; Gache et al., 1996; Smith et al., 1996; McLean et al., 1996]. That IFAP300 is related to plectin has previously been reported [Hermann and Wiche, 1987].

Guo et al. [1995] have used molecular genetics to generate a transgenic mouse lacking BP230. The hemidesmosomes in such mice lack the inner cytoplasmic most portion of the plaque [Guo et al., 1995]. Moreover, only wispy, nonbundled IF associate with these abnormal hemidesmosomes, implying that BP230 plays an important role in IF-cell surface anchorage. In contrast, here we

show that in a cell line lacking BP230, IF are found in association with α6β4 integrin [Hopkinson and Jones, 1994]. Indeed, our data indicate that this association is mediated by IFAP300. In this regard, hemidesmosomes lack IF association in the epidermal cells of patients with muscular dystrophy associated with epidermolysis bullosa simplex (MD-EBS) in which there is a loss of expression of HD1 (IFAP300?) protein [Gache et al., 1996; Smith et al., 1996; McLean et al., 1996].

How can we reconcile the results of our study as well as those which show a loss of IF connection to hemidesmosomes in the skin of MD-EBS patients with the data presented in Guo et al. [1995] concerning BP230 function [Gache et al., 1996; Smith et al., 1996; McLean et al., 1996]? One possibility is that both BP230 and IFAP300/HD1 play roles in IF-membrane anchorage in the same manner that multiple actin binding proteins (such as α-actin and talin) are involved in attachment of microfilament assemblies to the cell surface [Simon and Burridge, 1994]. Alternatively, BP230 may, in some way, modulate locally the organization of keratin bundles or individual keratin filaments in order to facilitate IFAP300/HD1-mediated IF interaction with the long cytoplasmic tail of the β4 integrin subunit.

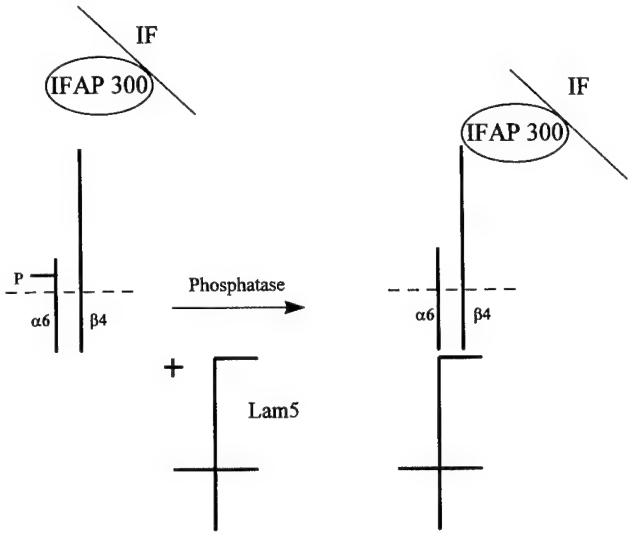

Fig. 13. This is a hypothetical scheme in which laminin-5 (Lam5) induces the association of IF with the cell surface via an interaction between IFAP300 and the cytoplasmic domain of the β4 integrin subunit. We also indicate in the diagram that dephosphorylation of the α6 integrin accompanies and may play a role in mediating this event. The dashed line represents the membrane of an epithelial cell. Laminin-5 is shown as an inverted t structure whose "G domain" interacts with the extracellular region of the α6β4 integrin heterodimer.

Assembly of Integrin-IF Complexes

Based on the results of this study, we hypothesize that dephosphorylation of the α6 integrin subunit, triggered by FG cell interaction with laminin-5, is necessary for the association of IFAP300 and keratin IF with the α6β4 integrin heterodimer (Fig. 13). This establishes an interaction, albeit indirect, between the laminin-5 of the extracellular matrix and the IF cytoskeleton. Indeed, in addition to its role in adhesion, the chain of molecules linking IF and laminin-5 is likely to play an important role in the transduction of signals from epithelial cells to the extracellular matrix and vice versa as suggested by the work of others [Mainiero et al., 1995; Baker et al., 1996]. The importance of such signals to epithelial cell function encourages us not only to pursue the identification of the phosphatase involved in the laminin-5-induced dephosphorylation of α6 integrin but also to investigate the possibility that IFAP300 binds directly to the long cytoplasmic domain of the β4 integrin subunit.

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Morphogenetic Effects of Soluble Laminin-5 on Cultured Epithelial Cells and Tissue Explants

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The rat cell line 804G assembles an extracellular matrix which induces not only the rapid adhesion and spreading of epithelial cells but also the assembly of a cell–matrix attachment device called the hemidesmosome. The major component of this matrix is laminin-5. We have purified rat laminin-5 from medium conditioned by 804G cells. Epithelial cells which are co-incubated with medium supplemented with soluble laminin-5 adhere and spread rapidly. Furthermore, human carcinoma cells undergo a dramatic morphologic change in the presence of laminin-5 and form orderly arrays resembling epithelial sheets. Soluble rat laminin-5 is selectively incorporated into an insoluble matrix of epithelial cells *in vitro*, since rat-specific laminin-5 antibodies stain cell–substrate contacts. Addition of medium containing soluble laminin-5 to explanted, human corneal rims induces assembly of hemidesmosomes, important cell–matrix attachment devices. Furthermore, rat-specific laminin-5 antibodies stain areas of contact between corneal epithelium and basement membrane, indicating that rat laminin-5 from the medium is incorporated into basement membrane. We discuss the use of laminin-5 as a medium supplement for the culture of both epithelial cells and epithelial tissue explants.

INTRODUCTION

Epithelial cells in tissues are separated from the underlying connective tissue by a structure called the basement membrane [1, 2]. The structural complexity of the basement membrane is mirrored by its variety of functions. For example, each basement membrane is composed of a mixture of matrix components including collagen, laminins, and heparan sulfate proteoglycan [2]. Embedded in this matrix are growth factors and various matrix degrading enzymes. As a unit these elements play an important role in regulating morphogenesis, adhesion, spreading, and motility of epithelial cells *in vivo* [3–8].

Under *in vitro* culture conditions many cells secrete basement membrane components and deposit them on their substrate. However, the repertoire of expression of extracellular matrix (ECM) proteins by cells *in vitro* is often limited. Critical components of natural basement membranes may be missing with a resulting negative impact on gene and protein expression. This problem is partially alleviated by the use of matrix-coated cell supports since this provides a more "normal" substrate for the proliferation and differentiation of cells [for example, see 4, 6, 9–11]. This, however, is only a temporary solution. First, the biological activities of immobilized matrix components may not entirely replicate that of their *in vivo* counterparts. Second, the natural turnover/degradation of matrix molecules requires regular removal of cells from their substrate so that they can be plated onto a fresh combination of matrix components. An obvious alternative would be to provide cells with soluble ECM proteins so that they can assemble their appropriate matrix. This is possible with fibronectin since it occurs in soluble form in plasma and can be appropriated by cells (e.g., from serum) for the assembly of a fibrillar extracellular meshwork [12]. However, this has rarely been assessed for basement membrane proteins such as the nonfibrillar laminins [13–15].

Recently, we characterized matrix elements manufactured and assembled by the rat bladder epithelial cell line 804G [16–19]. This matrix appears rich in rat laminin-5, composed of three subunits, one subunit of which has high amino acid sequence identity with the laminin-5 $\gamma 2$ subunit (previously termed laminin B2t), and one subunit having identity with the laminin-5 $\alpha 3$ subunit [16, 19, 20, 21].

The laminin-5 elements of the insoluble, assembled matrix of 804G cells are also found in the conditioned medium of these cells [18]. Moreover, Hormia *et al.* [18]

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have shown that incubation of cultured keratinocytes in 804G conditioned medium results in rapid cell attachment and assembly of hemidesmosomes. These workers assume, but do not directly demonstrate, that this response is due to the laminin-5 component of 804G conditioned medium. To further the latter studies, we have purified laminin-5 from 804G conditioned medium and analyzed the impact of culturing epithelial cells as well as corneal tissue explants in medium supplemented with this purified matrix molecule. Addition of purified soluble laminin-5 to the culture medium of epithelial cell lines induces both a dramatic rapid cell attachment and a spreading. Furthermore, we show that soluble laminin-5 can be incorporated into the insoluble matrix of carcinoma cells, human keratinocytes and, in addition, forming and formed basement membranes in explant material. Finally, we show that co-incubation of laminin-5 and corneal tissue results in an induction of hemidesmosome assembly by the epithelial cell components of the explants.

MATERIALS AND METHODS

Cell and explant cultures. 804G cells were cultured as previously reported [16]. At 4 days (1 day postconfluency) conditioned medium was removed from the cells, centrifuged at 1500*g* to remove cell debris, and then frozen at −20°C for later use. Immortalized human keratinocytes, HaCaT cells, were maintained as detailed in Hormia *et al.* [18]. FG cells were cultured as described by Kajiji *et al.* [22]. They are a metastatic pancreatic carcinoma cell line [22, 23]. Human eyes, stored on average for 4 days in a minimal medium called Optisol (Chiron, San Diego, CA), were procured from the Illinois Eye Bank.

Cell adhesion assays. Approximately 8×10^4 FG cells were plated into each well of a 96-well polystyrene microtiter plate (Starstedt, Inc., Newton, NC) in dilutions of 804G conditioned medium, control medium (MEM containing 10% fetal calf serum, CMEM), or medium supplemented with varying quantities of laminin-5, laminin-1, and fibronectin. After 45 min at 37°C, the plates were washed in PBS and adherent cells were fixed in 3.7% formaldehyde in PBS. The cells were then stained for 15 min at room temperature with 0.5% crystal violet in 20% methanol. The stained cells were washed extensively with water and solubilized in 1% SDS at 100 ml/well. Absorbance was measured on a Vmax microplate reader (Molecular Devices) at 570 nm.

Antibodies. A rabbit antiserum against the laminin-5 components of 804G matrix (J18) was prepared as detailed in Langhofer *et al.* [16] using matrix of 804G cells as immunogen (see below). A monoclonal antibody (5C5) against the 150-kDa polypeptide of 804G matrix was generated by injecting mice with 804G matrix. Spleen cells of immunized mice were fused with the mouse myeloma cell Sp2 [16]. 5C5 is of the IgG1 subtype and is specific for the α3 chain of rat laminin-5 [19]. The mouse IgG1 antibody TR1 against the γ chain of rat laminin-5 is described in detail in Plopper *et al.* [17]. Antibodies against laminin-1, fibronectin, and merosin (laminin-2) were obtained from GibcoBRL Life Technologies (Gaithersburg, MD). Horseradish peroxidase and fluorescein conjugated secondary antibodies were obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Protein preparations. Laminin-1 and fibronectin were purchased from Collaborative Biomedical Products (Bedford, MA). Merosin was purchased from GibcoBRL Life Technologies. Matrix of 804G cells was prepared according to Gospodarowicz [10]. In brief, confluent dishes of 804G cells were washed in phosphate-buffered saline (PBS), treated for 5 min with 20 m*M* NH_4OH, and then washed extensively in distilled water. The matrix was removed from the culture dish by solubilization in 8 *M* urea, 1% sodium dodecyl sulfate (SDS), in 10 m*M* Tris–HCl, pH 6.8, containing 10% β-mercaptoethanol. In the case of conditioned medium, 2 volumes of culture supernatant were added to 1 volume of 3× Laemmli type SDS sample buffer containing 10% β-mercaptoethanol, and then heated for 3 min at 95°C [24].

Laminin-5 was isolated from the medium of 804G cells by column chromatography. The medium contains approximately 1 mg/ml of laminin-5 (Fitchmun, unpublished observation). In brief, TR1 antibody was bound to Affi-Gel 10 (Bio-Rad, Hercules, CA). TR1-agarose was then packed into a C-Column (Pharmacia, Uppsala, Sweden). 804G medium was passed over the antibody column which had been equilibrated with 50 m*M* sodium phosphate, 100 m*M* NaCl, pH 7.7. The column was washed with five volumes of the same buffer and bound protein was eluted with 50 m*M* sodium phosphate, 50 m*M* diethylamine, 100 m*M* NaCl, pH 11.6. An equal volume of 100 m*M* sodium citrate, pH 5, was then added to the column eluate and the resulting solution was exchanged into 50 m*M* sodium phosphate, 100 m*M* NaCl, pH 7, via a G25 chromatography column (Pharmacia).

Immunoblotting. Protein samples, processed for gel electrophoresis on polyacrylamide gels, were transferred to nitrocellulose [25–27]. Immunoblotting was carried out as detailed previously in Zackroff *et al.* [27].

Immunofluorescence microscopy. Tissue pieces were rapidly frozen in liquid nitrogen and cryosections were prepared on a Tissue-Tek cryomicrotome. Sections were placed on microscope slides and then extracted for 5 min at −20°C acetone. After air drying they were incubated in primary antibody followed by fluorescein conjugated secondary antibody as detailed elsewhere [28]. Cells grown on glass coverslips were extracted for 3 min in −20°C acetone and then air dried. They were processed for fluorescence as described by Riddelle *et al.* [29]. Both sections and cells on coverslips were viewed in a Zeiss LSM10 confocal microscope.

Electron microscopy. Tissue was prepared for electron microscopy as detailed in Riddelle *et al.* [29]. Thin sections of embedded material were viewed in a JEOL 100CX electron microscope.

RESULTS

Plating of Epithelial Cells into Medium Supplemented with Laminin-5 Induces Rapid Adhesion and Cell Spreading

Medium conditioned by 804G cells is rich in laminin-5 (18; Fig. 1). The conditioned medium also contains fibronectin but little, if any, laminin-1 (Fig. 1). Hormia *et al.* [18] recently showed that when human keratinocytes are plated into 804G conditioned medium they rapidly attach to their substrate. To determine unequivocally whether this phenomenon is due to laminin-5 in the conditioned medium, we immunopurified laminin-5 from 804G conditioned medium using immunocolumn chromatography (Fig. 2). We then plated a variety of epithelial cells, including HaCaT keratinocytes as well as the pancreatic carcinoma line, FG, a highly metastatic epithelial cell, into medium supplemented with laminin-5. At various times after plating, cell adhesion and spreading were assessed morphologically by phase-contrast microscopy.

Within 45 min of plating, HaCaT cells rapidly ad-

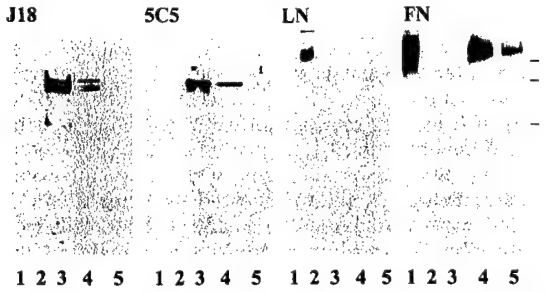

FIG. 1. Preparations of rat fibronectin (5 μg) (lane 1), rat laminin-1 (5 μg) (lane 2), deposited 804G matrix (10 μg) (lane 3), 804G cell conditioned medium (25 μg) (lane 4) and control medium (25 μg) (CMEM) (lane 5) were processed for immunoblotting using J18 serum, 5C5 antibodies, an antiserum against rat laminin-1 (LN), and an antiserum against rat fibronectin (FN) as indicated. Antibodies in the rabbit serum J18 recognize several major components of 804G deposited matrix migrating at 150, 140 135, and 100 kDa (J18, lane 3). The monoclonal antibodies 5C5 primarily recognizes a 150-kDa protein component in the matrix (5C5, lane 3). The 150-, 140-, and 135-kDa proteins recognized by J18 in the insoluble matrix laid down by 804G cells are also found in 804G cell conditioned medium (J18, lane 4). Similarly, the 150-kDa protein component of insoluble matrix protein that is reactive with 5C5 antibodies is found in 804G cell medium (5C5, lane 4). It should be noted that 804G conditioned medium contains some fibronectin (FN, lane 4) but no detectable laminin-1 (LN, lane 4). CMEM is not recognized by the 804G matrix antibodies (J18, lane 5; 5C5, lane 5). Rat fibronectin (FN, lane 1) and rat laminin-1 (LN, lane 2) are recognized by their appropriate antibodies. Dashes to the right of the last lane indicate molecular weights of 200, 150, and 100 kDa.

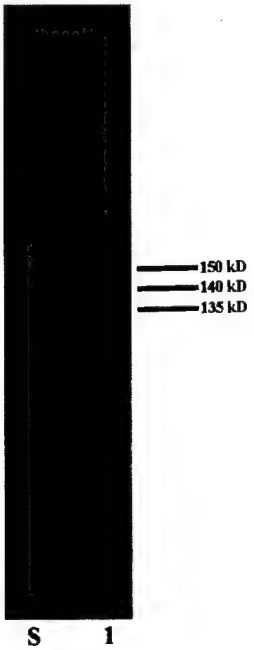

FIG. 2. Laminin-5 was purified from 804G conditioned medium. Lane S shows molecular weight standards of 200, 116, 97, and 66 kDa. Lane 1 shows the SDS–PAGE profile of purified laminin-5 (3 μg was run in a single gel lane). It is composed of three major polypeptides of 150, 140, and 135 kDa.

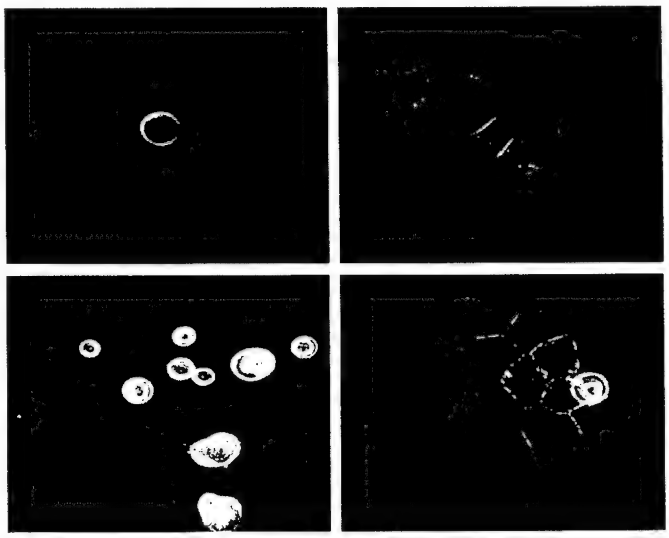

FIG. 3. HaCaT cells (A, B) or FG cells (C, D) were plated onto glass coverslips in control medium (A, C), or medium supplemented with 5 μg/ml of laminin-5 (B, D). The cells were then viewed by phase-contrast microscopy after 45 min (A, B, D) or 24 h (C). HaCaT cells are poorly adherent to their substrate at 45 min when plated onto glass (A) but are well spread out onto the coverslip when plated into laminin-5-containing medium (B). Even after 24 h on glass, FG cells are clumped together (C). In contrast, at 45 min after plating into laminin-5-containing medium, the FG cells are well adhered to their substrate, appear spread out, and are grouped together as a sheet (D). Bar, 25 μm.

here and, most remarkably, assume a flattened morphology when "co-incubated" with laminin-5 (Fig. 3A). In their normal medium or medium supplemented with laminin-1, laminin-2, or fibronectin, HaCaT cells show poor attachment to their substrate (Fig. 3B; only the result for the normal medium experiment is shown). This is consistent with the data of Hormia *et al.* [18]. We chose laminin-2 as one of our test matrix proteins based on a recent report that it has a dramatic impact on cell adhesion of fibroblastic cells [30]. However, it had no obvious impact on adherence or spreading of HaCaT cells.

FG cells show a more dramatic morphological change when plated into laminin-5-containing medium. In their normal medium or medium containing 5 μg/ml laminin-1, laminin-2, or fibronectin, FG cells plated onto glass coverslips or tissue culture plastic adhere poorly even after 24 h (Fig. 3C; only the result for the normal medium experiment is shown). Indeed, the FG cells prefer to aggregate together and have a rounded morphology (Fig. 3C). Impressively, 45 min after plating into medium containing 5 μg/ml of soluble laminin-5, FG cells appear fully spread and organized into squamous sheets (Fig. 3D).

We next quantitated the impact of laminin-5-supplemented medium on HaCaT and FG cell-substrate attach-

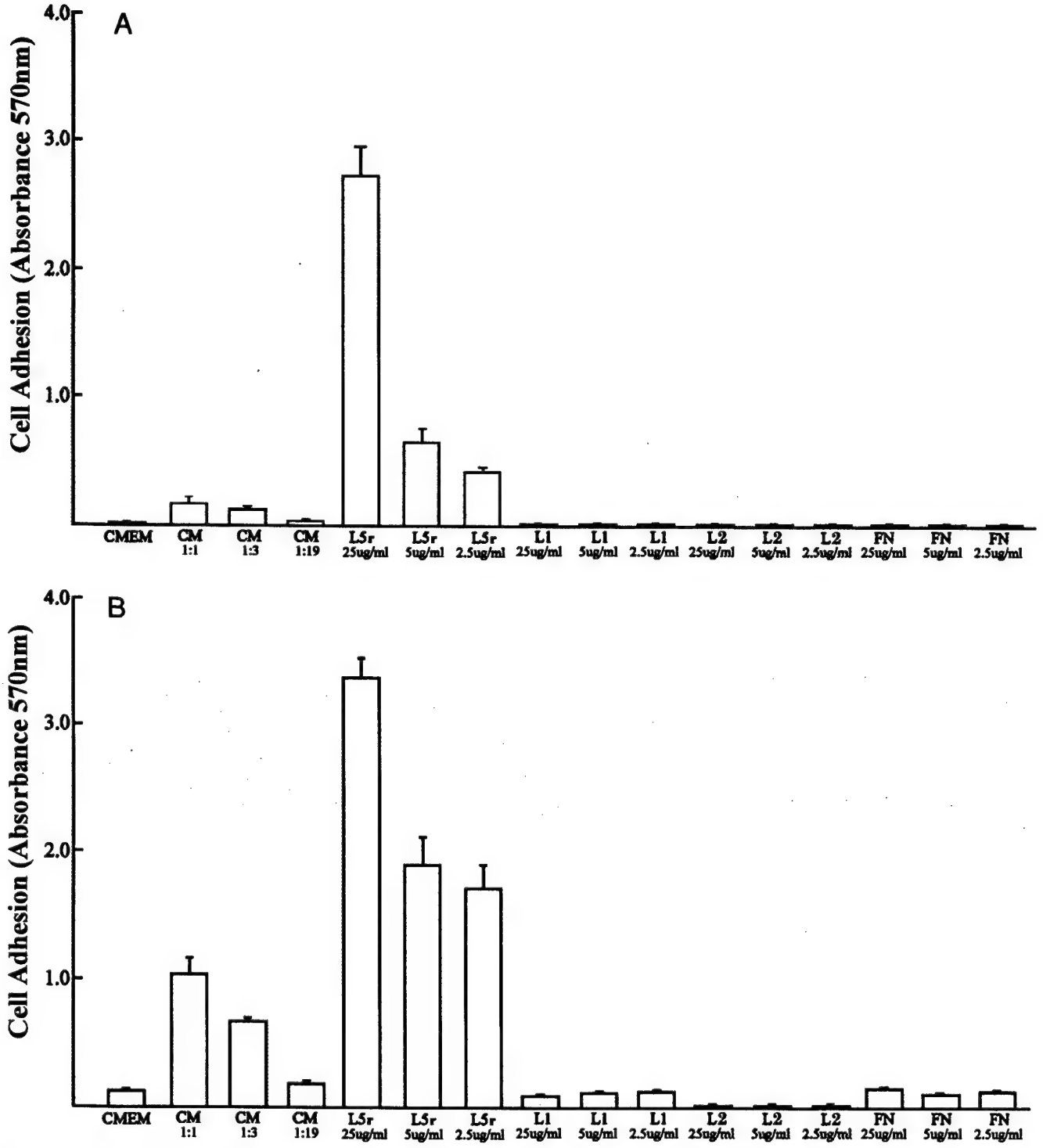

FIG. 4. Rapid adhesion of HaCaT (A) and FG (B) cells in control medium (CMEM) and 804G conditioned medium (CM), rat laminin-5 (L5r), laminin-1 (L1), laminin-2 (L2), and fibronectin (FN) supplemented medium was quantified at 45 min after plating. Concentrations of matrix proteins and dilutions of 804G conditioned medium (the latter were made in CMEM) are indicated along the *x*-axis. Data shown are means (±SD).

ment in a rapid adhesion assay (Fig. 4A). In these assays, we compared laminin-5 with laminin-1, laminin-2, and fibronectin. Medium supplemented with the latter matrix components at concentrations up to 25 μg/ml fails to support rapid adhesion of FG and HaCaT cells while purified laminin-5 induces rapid attachment of both cell types at

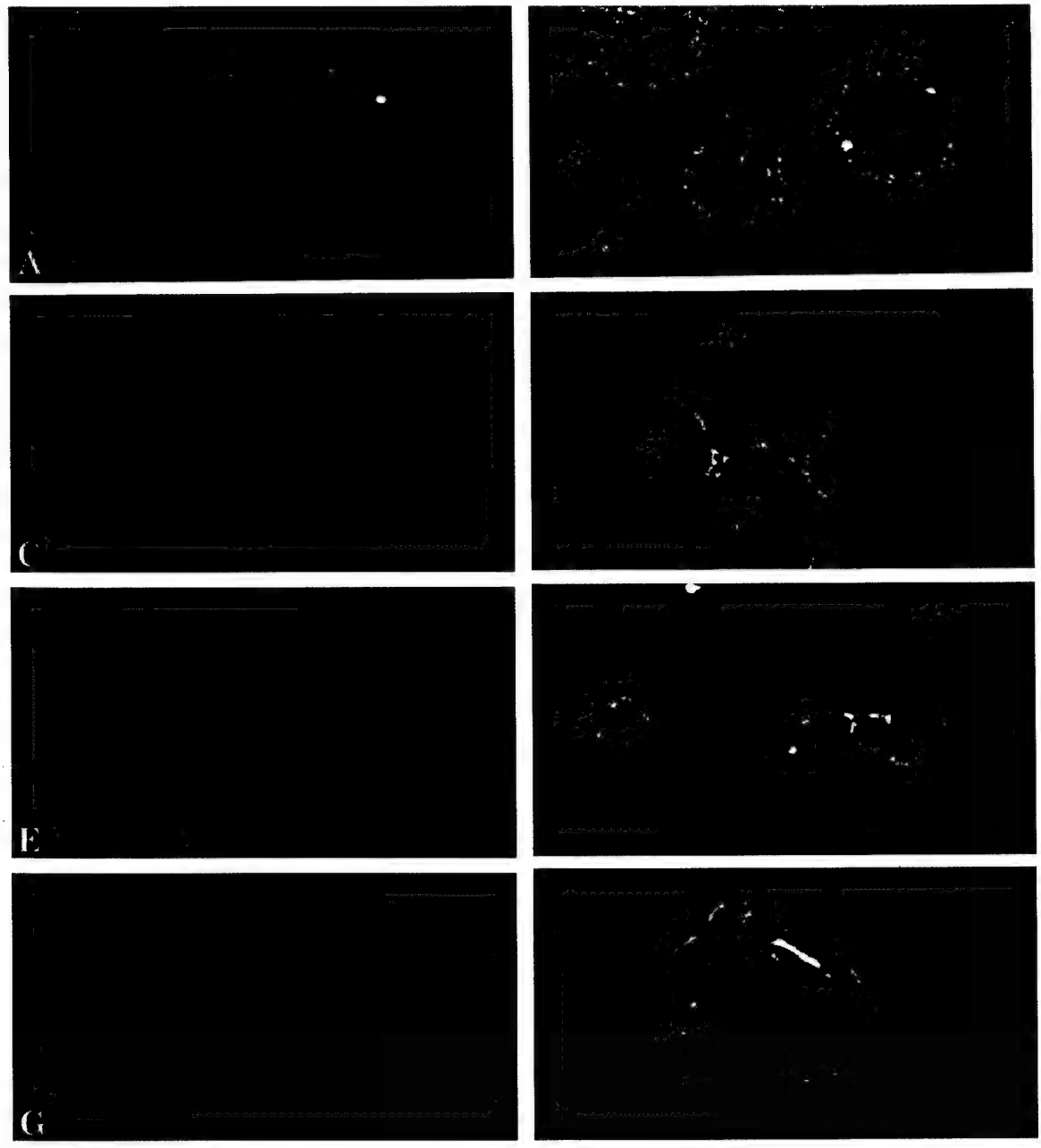

FIG. 5. HaCaT cells (A, B) and FG cells (E, F) were plated into medium supplemented with 5 μg/ml of laminin-5 and, 24 h later, were processed for immunofluorescence microscopy using 5C5 antibodies (A, E). In A and E, 5C5 antigen appears as large discrete circles that lie toward the edge of each cell, along regions of the cell–substrate interface as determined by confocal microscopy. In C and G, 5C5 antibody staining of HaCaT cells and FG cells, which had been plated into their normal medium on glass coverslips allowed to adhere to glass coverslips for 24 h, is shown. In C and G, 5C5 antibody fails to stain FG and HaCaT cells. (B, D, F, H) Phase-contrast images of the cells. Bar, 10 μm.

concentrations as low as 2.5 μg/ml (Fig. 4A). It is important to note that medium supplemented with 2.5 μg/ml laminin-5 is able to support rapid adhesion of HaCaT and FG cells more effectively than 804G conditioned medium (Fig. 4A). This result is consistent with the estimated concentration of laminin-5 in 804G conditioned medium (1 μg/ml) (Fitchmun, unpublished observation). Together, our morphological and adhesion assay results indicate that laminin-5 is the component of 804G conditioned medium responsible for the rapid adhesion and spreading effect on HaCaT and FG cells.

Morphological Evidence That Soluble Laminin-5 Is Utilized by Human Epithelial Cells

To gain independent evidence that soluble laminin-5 supports cell adhesion, we next investigated the in-

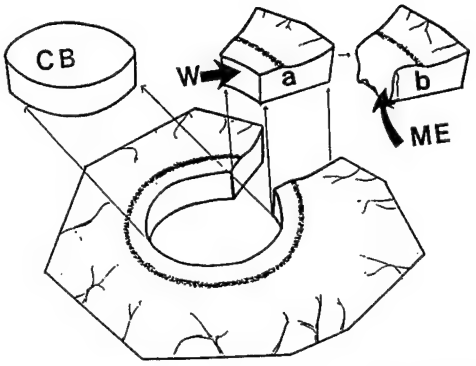

FIG. 6. Diagrammatic representation of human corneal rims used in our analyses. In all of our material a central corneal button (CB) had been removed for transplantation. The residual rim has a "wounded" (W) side resulting from removal of the CB (wedge a). Cells will reepithelialize the wound *in vitro* after several days (wedge b). ME, migrated epithelium.

corporation of laminin-5 from medium into areas of contact between adhering/spreading cells and substrate. The monoclonal antibody 5C5 recognizes an epitope of the α3 chain of laminin-5 which is species specific since 5C5 reacts with rat but not human basement membranes in immunofluorescence and immunoblotting analyses [16, 19, 31; Langhofer and Jones, unpublished results]. Therefore, 5C5 can be used to monitor the utilization of laminin-5 by human cells incubated in the laminin-5 supplemented medium, without interference from any laminin-5 which may be made endogenously by such human cells.

After the HaCaT and FG cells were plated onto glass coverslips in medium containing soluble laminin-5, 5C5 antibodies become reactive with large discrete circles lying toward the edge of each cell, along regions of the cell–substrate interface as determined by confocal immunofluorescence microscopy (Fig. 5). 5C5 antibody shows no reactivity with HaCaT and FG cells incubated in control medium (Fig. 5).

Laminin-5 Capture by Epithelial Cells in Tissue Explants

The effects of laminin-5-containing medium on morphology, adhesion, and spreading of cultured epithelial cells suggests a possible use in the storage and maintenance of epithelial tissue explants. For example, donor human corneas are routinely stored in a minimal, serum-free medium ("Optisol") for several days prior to transplantation. After 24 h of storage, there is a complete disappearance of formed hemidesmosomes in the basal cells of the corneal epithelium of the rims (see below).

We obtained human corneal rims from penetrating keratoplasty. All rims had been maintained for at least 24 h in Optisol and thus lacked hemidesmosomes. Each

FIG. 7. Electron micrographs of human corneal material maintained for 72 h in the presence of either control medium (CMEM) (a), 5 mg/ml laminin-1 (b), or 5 mg/ml laminin-5 (c). No hemidesmosomes are present in the "unwounded" portion of the rims in a and b, along the site of epithelial cell/basement membrane zone interaction (arrowheads). In comparison, there are numerous hemidesmosomes in the unwounded region of the rim in c (arrowheads). The large arrowhead in c indicates a hemidesmosome shown at higher magnification in the inset. It possesses a subbasal dense plate (arrowheads). Bars in a, b, and c, 1 μm; bar in inset, 50 nm.

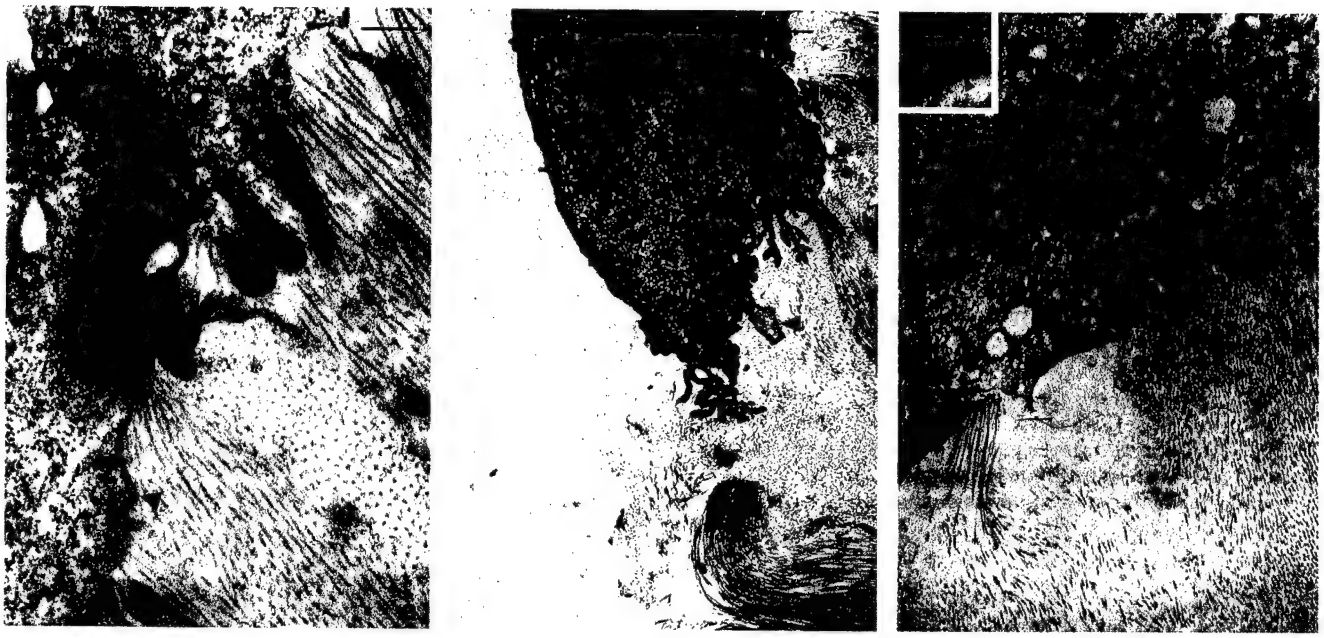

FIG. 8. Electron micrographs of the "wounded" portions of human corneal material maintained for 72 h in the presence of control medium (CMEM) (a), 5 mg/ml of laminin-1 (b) or 5 mg/ml of laminin-5 (c). Regions of epithelial cells which have migrated over the wounded sides of the corneal rims are shown (see Fig. 6). In a and b, there are no hemidesmosomes at sites of epithelial cell–stromal interaction (arrowheads). In contrast, hemidesmosomes are observed at comparable sites in c (arrowheads). One of these, indicated by the large arrowhead, is shown in the inset in c. Note the subbasal dense plate (arrowhead) of this hemidesmosome. Bars in a, b, and c 1 μm; bar in inset, 50 nm.

rim, consisting of peripheral corneal and limbal epithelial cells with underlying connective tissue, had an "unwounded" region with intact epithelium, and a cut or "wounded" connective tissue (stromal) side (Fig. 6). Epithelial cells from the unwounded region of the rims will eventually cover such a wound surface provided the rims are cultured in serum-containing medium rather than Optisol (Fig. 6).

Three human corneal rims were cut in half. One half of each was maintained in medium supplemented with soluble laminin-5, while the other half was maintained in laminin-1 supplemented medium. Three rims were maintained in control medium (CMEM) alone. After 72 h each of the corneal rim samples was further divided, one portion being processed for electron microscopy while the other portion was frozen in liquid nitrogen prior to processing for immunofluorescence microscopy.

In those rims maintained in CMEM alone and medium supplemented with laminin-1, there are no hemidesmosomes along the site of epithelial cell–stromal interaction in the unwounded tissue area (Figs. 7a and 7b). Furthermore, corneal epithelial cells which migrate over the wounded sides of such rims fail to assemble any hemidesmosomes (Figs. 8a and 8b). Indeed, incubation of rims in laminin-1 supplemented medium inhibits migration over the sides of the rims (Fig. 8a). In sharp contrast, numerous hemidesmosomes are found both along the unwounded and wounded sides of the rims maintained in medium supplemented with laminin-5 (Figs. 7c and 8c). These structures possess all of the defining morphological characteristics of hemidesmosomes including a subbasal dense plate located in the lamina lucida region of the basement membrane and a tripartite cytoplasmic plaque to which keratin intermediate filaments adhere (insets in Figs. 7c and 8c; [31]).

To assess the fate of rat laminin-5 from the medium in this system the human corneal rims were processed for immunofluorescence using 5C5 antibodies. An intense band of fluorescence is observed at the interface of epithelium and connective tissue in the human corneal rims incubated in laminin-5-containing medium, while the corneal rims incubated in CMEM are negative (Figs. 9a, 9c, and 9e). The 5C5 antibody staining is observed both at the top, unwounded portion of the rims, as well as along the area where epithelial cells have established new contact with the wounded connective tissue (Figs. 9c and 9e). This pattern of staining implies that laminin-5 is incorporated into both preexisting and newly formed basement membranes.

DISCUSSION

Hormia *et al.* [18] previously showed that when HaCaT cells are cultured in the presence of 804G conditioned medium they rapidly adhere. In addition, if lam-

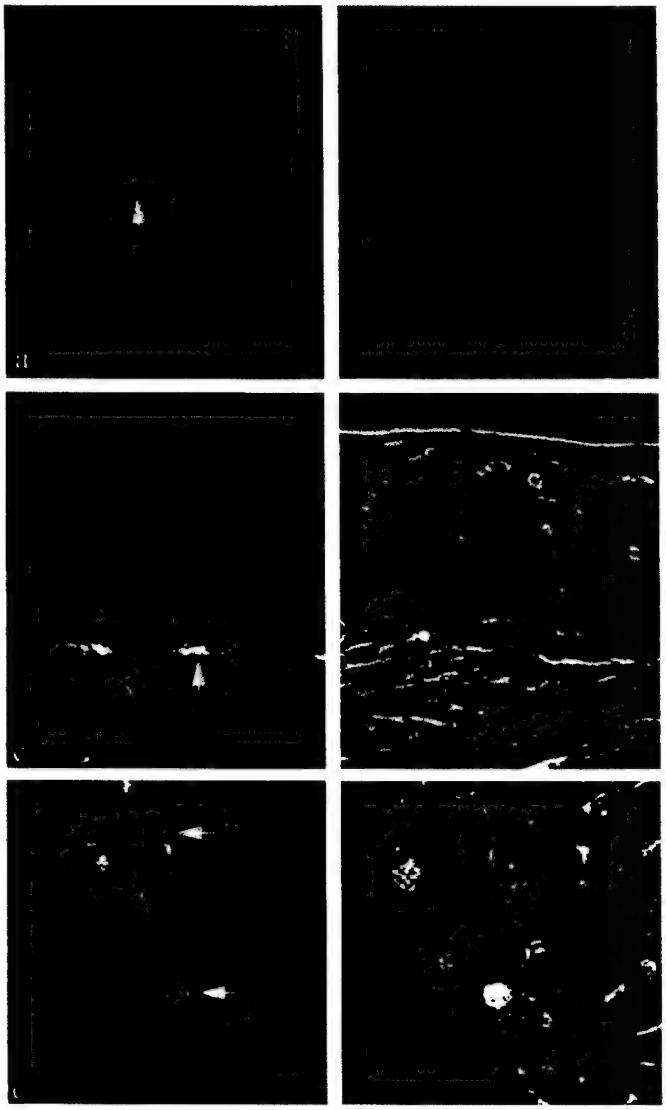

FIG. 9. Human corneal tissue material was processed for immunofluorescence microscopy using 5C5 antibody. No staining of corneal rims incubated for 72 h in control medium is seen (a; the arrow marks the epithelial–stromal interface). There is an intense fluorescence (arrow) at the region of epithelium–connective tissue interaction in the unwounded region of human corneal rims incubated for 72 h in medium containing 5 mg/ml laminin-5 (c, arrow). In addition, the region where epithelial cells have established contact with the "wounded" connective tissue of the rim (see Fig. 6) (arrows to the right) is stained by 5C5 antibodies in rims incubated for 72 h in laminin-5-containing medium (e). Phase images of the tissue sections are shown in b, d, and f. Bar in b, 35 μm.

inin-5 is absorbed from 804G conditioned medium using a laminin-5 polyclonal, polyspecific antibody, then the ability of the medium to induce rapid cell attachment is abolished. Thus the laminin-5 component in the 804G medium is the likely agent that induces the the rapid attachment of HaCaT cells reported by Hormia *et al.* [18]. Here we provide direct evidence for the latter by showing that addition of purified soluble laminin-5 to medium of cultured epithelial cells, in particular FG and HaCaT, is capable of inducing their rapid attachment and spreading. Moreover, we also show that addition of laminin-5 to the medium of FG cells has a dramatic morphological impact on the cells. The FG carcinoma cells, which tend to grow in mounds and fail to flatten onto plastic substrates under normal culture conditions, spread rapidly in the presence of laminin-5 and resemble "normal" epithelial cells. This remarkable morphogenetic change involves the "capture" by the cells of laminin-5 components, since intense immunostaining with antibody 5C5 is observed toward the edges of flattening HaCaT cells. We show a similar phenomenon with FG cells. The antigen recognized by the 5C5 antibodies must originate from the medium since it is rat-specific and is therefore incapable of recognizing laminin subunits secreted by the human epithelial cells we have used.

In both HaCaT and FG cells plated in the presence of laminin-5, the 150-kDa (α chain) of laminin-5, visualized using 5C5 antibodies, is localized at sites of cell–substrate interactions, in characteristic ring-like structures. How are these ring structures produced by the cells? One possibility is that the FG cells actively recruit laminin-5 from the laminin-5-supplemented medium and concentrate it at adhesion sites. Alternatively, laminin-5 may adhere to plastic and glass spontaneously, forming a high-affinity substrate to which epithelial cells adhere. If this is the case, then the human cells in some way reorganize the matrix to generate the rings of laminin-5 reactive material. Obviously, this mechanism needs to be explored in detail, because it will probably yield insights not only into the morphogenetic influence of laminin-5 on epithelial and carcinoma cells but also the manner in which basement membranes are assembled *in vivo.*

Our experiments with HaCaT and FG cells illustrate an important aspect of our study on laminin-5, i.e., we simply add soluble laminin-5 to cell or tissue cultures. This approach is distinct from that detailed by Rousselle and Aumailley [11] who have described aspects of attachment of epithelial cells to human laminin-5 coated substrates. Indeed, we show here that laminin-5 is active in a soluble form and can be simply used as a media supplement, obviating the need for preparing laminin-5 coated substrates prior to addition of cells.

The use of laminin-5 as a media supplement is not limited to cultured cell lines. In corneal tissue explants maintained in laminin-5 supplemented medium, laminin-5 is incorporated into forming as well as preexisting basement membranes. Moreover, when corneal pieces are incubated in medium supplemented with laminin-5 assembly of hemidesmosomes, epithelial cell–basement membrane adhesion devices of the cornea epithelium, is induced. Indeed in explant cultures, the overall

effect of laminin-5 supplemented medium is in the efficient maintenance of epithelial attachment resulting in better preservation of tissue integrity. Whether this is peculiar to laminin-5 or will be a property of other, as yet uncharacterized, laminins remains to be seen.

804G cells are an obvious excellent source of soluble laminin-5. Others have shown that normal keratinocytes also secrete laminin-5 into their medium and that soluble laminin-5 is a component of amniotic fluid [32–34]. Can soluble laminin-5 be incorporated into the matrices of cells and tissues *in vivo,* possibly during development or wound repair? We do not yet know the answers to this question. Further analyses of the function of laminin-5 and its regulation will be needed before we can begin to tackle such intriguing issues.

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Structure and Assembly of Hemidesmosomes

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Summary

The hemidesmosome is a complex junction containing many proteins. The keratin cytoskeleton attaches to its cytoplasmic plaque while its transmembrane elements interact with components of the extracellular matrix. Hemidesmosome assembly involves recruitment of α6β4 integrin heterodimers as well as cytoskeletal elements and cytoskeleton-associated proteins to the cell surface. In our cell culture models, these phenomena appear to be triggered by laminin-5 in the extracellular matrix. Cell interaction with laminin-5 apparently induces both phosphorylation and dephosphorylation of subunits of α6β4 integrin. There is emerging evidence that such events are necessary for subsequent cytoskeleton anchorage to the hemidesmosome cytoplasmic plaque. Once assembled the hemidesmosome plays an essential role in maintaining firm epithelial adhesion to the basement membrane with hemidesmosome disruption being a hallmark of certain devastating blistering diseases. However, the hemidesmosome is more than just a stable anchor since it is also the site of signal transduction, mediated by its α6β4 integrin component. This review discusses our current knowledge of the structure and assembly of the hemidesmosome.

Introduction

Epithelial tissues maintain stable connection to basement membranes, and the dermal tissue below, via adhesive interactions between the cells in the basal layer of the epithelium and extracellular matrix proteins in the basement membrane. In many epithelia, there are specific points of connection, termed hemidesmosomes, which

take the form of specialized transmembrane cell-matrix junctions between the epithelial cells on one hand and the basement membrane on the other (Fig. 1)(1,2). The name for these structures derives from their appearance in the electron microscope as half desmosomes, since both the desmosome, a cell-cell junction, and the hemidesmosome have similar multi-layered electron-dense cytoplasmic plaques to which keratin bundles attach (Fig. 1)(1). In this review we will describe the structure of the hemidesmosome, its molecular composition and what is known about its assembly. We will concentrate our survey on recent papers relating to the role of integrins and matrix components in hemidesmosome formation. Such analyses have been facilitated by the identification of cell lines which assemble hemidesmosomes readily and rapidly in vitro. One of these lines, termed 804G, which we identified a number of years ago, has been used extensively by groups around the world to study hemidesmosome protein-protein interactions as well as hemidesmosome assembly (3-8). In addition, we will discuss recent studies of human skin diseases whose pathogenesis involves hemidesmosome components. These studies have also provided valuable clues to the role of integrins and matrix in hemidesmosome formation (2). We will not discuss the extensive, older literature on hemidesmosome assembly during wound healing and in various tissue models.

Hemidesmosome Functions

It has long been assumed that hemidesmosomes play an important role in establishing and maintaining adhesion of an epithelial tissue to the basement

membrane, based on the appearance of hemidesmosomes in the electron microscope: they look like spot welds (Fig. 1)(9). This assumption has gained support following the discovery that perturbation in hemidesmosomal integrity is a feature of a number of skin diseases which are characterized by epithelial cell/basement membrane dysadhesion and blister formation (10-24). These skin diseases include the autoimmune diseases bullous pemphigoid (BP) and citricial pemphigoid (CP) as well as a group of genetic diseases, grouped under the terms junctional epidermolysis bullosa (JEB) and generalized atrophic benign epidermolysis bullosa (GABEB). In addition, since hemidesmosomes are absent from those epithelial cells which are populating wounds as well as those epithelial tumor cells which are invasive, it has been suggested that hemidesmosomes are required for stable epithelial cell anchorage and that their loss is a necessary prerequisite for cell motility (see, for example, 25-27).

We would like to emphasize, however, that hemidesmosomes are more than just spot welds. There are emerging data that they are involved in both inside-out and outside-in signaling phenomena and may therefore affect such diverse cellular activities as gene expression, cell proliferation, and differentiation (7,28). In addition, they apparently play an important role in morphogenetic events including those involved in breast epithelial tubule formation (29). At the molecular level, all of the processes in which hemidesmosomes participate are ultimately related to the individual protein components of the structure. These will be described next.

Hemidesmosome Structure and Protein Composition

In the electron microscope, a hemidesmosome can be seen as having a triangular-shaped cytoplasmic plaque, a sub-basal dense plate just below and external to the plasma membrane, thin extracellular anchoring filaments which extend from the plate into the basement membrane, and thicker anchoring fibrils made of condensed collagen VII dimers, which reach down into the dermis (Fig. 1)(1,9). Hemidesmosomes mediate attachment of basal cells to the basement membrane thereby providing a connection between the cytoskeletal elements of the cell to the proteins in the extracellular matrix which constitute the basement membrane. The principal agent for accomplishing integration of the cytoskeleton and extracellular matrix at the site of the hemidesmosome is the integrin heterodimer, $\alpha 6\beta 4$ (Fig. 2)(1,2). The $\alpha 6$ and $\beta 4$ integrins associate non-covalently and traverse the plasma membrane. In the cytoplasm, the unusually long tail of the $\beta 4$ integrin subunit likely binds cytoplasmic proteins, such as IFAP300 (a 300kD intermediate filament associated protein), plectin and HD1, which mediate association between the hemidesmosome and the keratin cytoskeleton (Fig. 2)(30-33). Whether HD1, plectin and IFAP300 are one and the same protein remains controversial although a variety of immunological procedures suggest that they are (21,34). The integrin $\alpha 6\beta 4$ also serves as a transmembrane receptor for an extracellular matrix molecule called laminin-5, and together the $\alpha 6\beta 4$ integrin/laminin-5 complex not only provides the core of hemidesmosome structure but is believed to be the conduit for hemidesmosome-mediated cell signaling (1,2,7,28,35-37). For example, the

cytoplasmic domain of the β4 subunit has been shown to associate with a number of molecules involved in signal transduction including Shc and Grb2 and is linked to the p21 pathway which regulates cell cycle (7,28,36).

In addition to α6β4 integrin, there is another transmembrane protein of the hemidesmosome, the bullous pemphigoid antigen II (BP180, BPAG2 or type XVII collagen)(Fig. 2)(38-42). This 180kD protein is recognized by autoantibodies in the sera of patients afflicted with the blistering skin disease, BP. BP180 is a type II transmembrane protein with an extracellular domain consisting of a series of GLY-X-Y repeats, hence its designation in the collagen family (39-41). BP180 assembles as a trimer and is believed to contribute to the structure of the anchoring filament (1,43). Using molecular genetic approaches, it has been shown that BP180 associates with β4 integrin in the cytoplasm and with α6 integrin extracellularly (6,8). Indeed, BP180 may be a second ligand for the α6β4 integrin (44)

Another BP antigen (BP230, BPAG1), a component of the hemidesmosome plaque, is involved in linkage of keratin cytoskeleton to the hemidesmosome (Fig. 2)(28,45). Like BP180, BP230 was first discovered as the antigen of autoantibodies in the sera of BP patients. The C-terminus of BP230 is capable of binding keratin IF and mice that lack BP230 have poorly formed hemidesmosomes to which few, if any, keratin filaments attach (45). In this regard, certain tumor cells express an isoform of BP230 which lacks the keratin binding domains of the BP230 C-terminus (46). This has led to speculation that expression of this truncated isoform rather than the intact BP230 molecule may allow a cell to regulate hemidesmosome assembly (46).

Circumstantial data suggest the possibility that BP230 interacts with the cytoplasmic domain of BP180 (44). In particular, in cultured cells in which BP180/α6 integrin association has been inhibited, BP230 retains its cell surface co-localization with BP180 but not with α6β4 integrin (44). The possibility that BP180 mediates keratin-cell surface interaction via an association with BP230 is indicated diagrammatically in Fig. 2.

The α6β4 integrin heterodimer, as well as laminin-5, are expressed in cells which, in the body, do not possess hemidesmosomes (see, for example, 47,48). Such is the case in the simple epithelial cells of the gut. Some have argued that the α6β4 integrin heterodimers under such circumstances are incorporated into junctions which they have called type II hemidesmosomes (48). Since these structures are not well defined and do not have the complex ultrastructure of the epidermal hemidesmosome, we will not consider type II hemidesmosomes here.

Hemidesmosome assembly

Role of Integrins and BP180 (type XVII collagen)

Previous work done in our laboratory and others has demonstrated that the α6β4 integrin pair is essential for the nucleation of hemidesmosome assembly. 804G cells which normally form hemidesmosomes in culture lose this ability when plated in the presence of antiserum against α6β4 (4). In fact, antibodies to β4 alone disrupt established hemidesmosomes, and α6 antibodies prevent adhesion and even induce cell detachment (25). That α6β4 integrin is involved in nucleation of hemidesmosome

assembly has been confirmed by molecular genetic studies in cultured cells (49). For example, forced expression in 804G cells of a tail-less β4 integrin, a mutant β4 lacking most of its large cytoplasmic domain, has a dominant negative effect on hemidesmosome assembly (49).

Furthermore, keratinocytes of transgenic mice lacking β4 integrin expression are unable to assemble any hemidesmosomes, and detachment of their epithelial tissues from the dermis results (50,51). Similarly, transgenic mice in which α6 integrin expression has been knocked out develop skin blistering, apparently because of an absence of hemidesmosomes, and the mice die at the neonatal stage (52). In addition, hemidesmosomes in the skin of human patients suffering from a particular form of JEB associated with pyloric atresia are abnormal, with most, if not all of them lacking well formed cytoplasmic architecture and sub-basal dense plates. In some patients this is apparently the result of a mutation in the β4 integrin gene while in others α6 integrin is mutated (17,20,24). In both instances, the mutations result in premature termination of transcription of the mutated gene.

Two studies indicate that the phosphorylation state of both α6 and β4 integrins may be crucial to their ability to nucleate hemidesmosome assembly and to recruit cytoskeleton elements to the cell surface. In the case of α6 integrin, ligand (laminin-5) occupancy of α6β4 integrin heterodimer "triggers" a dephosphorylation of serine residue 1041 in the cytoplasmic domain of the α6 integrin subunit (33). Dephosphorylation of α6 integrin correlates temporally with a reorganization of the keratin cytoskeleton such that keratin bundles and their accessory proteins become

associated with the cell surface at sites rich in dephosphorylated α6 integrin subunits (33). These sites are also where the cell interacts with laminin-5 (33). On the other hand, β4 integrin is tyrosine phosphorylated when the α6β4 heterodimer is crosslinked by β4 antibodies or upon α6β4 interaction with laminin-5 (7). Moreover, certain sites of tyrosine phosphorylation in the β4 cytoplasmic domain are apparently necessary for β4 integrin incorporation into hemidesmosomes (7).

The function of α6β4 integrin heterodimers and, in particular, their role in nucleation of assembly of hemidesmosomes can be modulated not only by laminin-5 but also, possibly, by BP180. A recent report has provided evidence that the interaction between BP180 and α6 integrin occurs via a stretch of amino acids in the short non-collagenous extracellular domain (NCE) of BP180 (44). The NCE of BP180 lies close to the membrane spanning region of the molecule, contains several charge residues and is the target site of pathogenic autoantibodies in BP (11,12). Hopkinson et al. (44) have shown that a 14 mer peptide whose sequence is contained within this region perturb hemidesmosome assembly in cultured cells.

It should be noted that a role for BP180 in hemidesmosome assembly is also suggested by work looking at the organization of the epidermis in a subset of patients with the blistering disease, GABEB, in which there are mutations in the gene encoding BP180 (18,23). In some patients a mutation creates a premature termination of transcription of the BP180 gene and an absence of BP180 protein in the epidermis (18). In others a mutation induces a splice defect which has been proposed to destabilize BP180 protein or inhibits the assembly of the BP180 trimer (23). In the

basal epidermal cell layers of the skin from such patients both α6β4 integrin and laminin-5 are apparently normally distributed at sites of cell-basement membrane interaction (18,23). However, hemidesmosomes in the diseased skin are either absent or lack well developed cytoplasmic plaques.

Interestingly, a protein migrating around 120kD in SDS-PAGE, has recently been shown to be immunologically related to BP180 (53). This protein is recognized by antibodies in the sera of linear IgA dermatosis (LAD) patients and is a component of the anchoring filaments of the hemidesmosome (38,54). Monoclonal antibodies against the bovine form of the LAD protein inhibit hemidesmosome assembly and induce disruption of epithelial cell-basement membrane attachment in an in vitro model of corneal wound healing (25). Whether the LAD antigen is a proteolytic product of BP180, possibly encompassing the extracellular BP180 domain since it is found in the conditioned medium of keratinocytes, or whether it is produced by alternative splicing of BP180 message is still unknown. Nonetheless, the LAD antigen apparently plays a role in assembly as well as in the maintenance of the structural integrity of the hemidesmosome.

Role of Laminin-5

The mechanisms by which hemidesmosomal proteins are mustered to the assembling hemidesmosome at the basal aspect of the cell are not yet defined. Indeed, we are only beginning to understand how the integrins themselves are first

recruited, so that the hemidesmosome may form around them. An important clue relates to the extracellular ligand for which α6β4 is a receptor: laminin-5 (35).

Like other laminin family members, laminin-5 is a heterotrimeric molecule with a cruciform shape (37,55). Each laminin-5 molecule consists of α3, β3 and γ2 subunits (56). All three subunits are smaller than their counterparts in laminins-1 through -4. Of note, however, is that like all other laminins described to date, laminin-5 possesses a characteristic large globular, or G domain, composed of the C-terminus of the α3 subunit (37,55,56).

Laminin-5 is synthesized initially as a 460 kD molecule, which undergoes specific processing to a smaller form after being secreted into the extracellular matrix (57-59). The size reduction is a result of processing of the α3 and γ2 subunits from 200-190 to 160 kD and from 155 to 105 kD respectively (57-59). Laminin-5 is abundant in transitional epithelium, stratified squamous epithelia, and other epithelial glands, all of which possess hemidesmosomes, although it is also expressed in tissues such as lung mucosa that do not (29,47).

Laminin-5 is the major element of the matrix secreted by cells, such as 804G and MCF-10A, that have the ability to assemble hemidesmosomes in vitro (3,29,60)(Fig. 3). Furthermore, hemidesmosome elements and laminin-5 co-distribute along the substrate attached surfaces of these cells (37). Our laboratory has used 804G cells to assess the role of laminin-5 in hemidesmosome assembly. In initial studies, we used a well established technique to prepare the laminin-5 rich matrix which is deposited onto substrate by 804G cells (61). In this procedure, 804G

cells are lysed and then cell remnants are blown off the matrix using a combination of NH_4OH and water. A test cell line is then plated onto the denuded matrix and after 24 hr. the cells are processed for both light and electron microscopy. Using this procedure, we have shown that the laminin-5 component of the matrix of 804G cells determines hemidesmosome protein organization in the test cells (37,60)(Fig. 4). At the ultrastructural level, we have quantified hemidesmosome assembly (60). Not only do epithelial cells rapidly adhere to the laminin-5 rich matrix of 804G cells but a number of normal and tumor cells are induced to assemble hemidesmosomes (26,37,60,62). In this system, adhesion of epithelial cells to laminin-5 rich 804G matrix can be inhibited by a combination of α3 integrin and α6 integrin blocking antibodies (Fig. 5). However, α6β4 integrin/laminin-5 ligation which leads to hemidesmosome assembly, but not adhesion, can be inhibited by the α6 integrin antibody GoH3 alone (Figs. 4 and 5). Based on their study of keratinocyte adhesion to laminin-5, Carter and his co-workers have suggested that the α3β1 integrin pair mediates initial attachment of epithelial cells to laminin-5, after which ligation is transferred to α6β4 integrin, by an unknown mechanism, which then mediates long-term stable adhesion via induction of hemidesmosome assembly (63). However, we emphasize that α3β1 integrin is not necessary for hemidesmosome assembly in vivo since epidermal cells assemble relatively normal appearing hemidesmosomes in the skin of transgenic mice lacking α3 integrin (64). This is consistent with a recent report from Xia et al. (65). These workers have shown that, under circumstances

when the function of the α3β1 integrin heterodimer is suppressed, α6β4 integrin alone can mediate anchorage of epidermal cells in culture to laminin-5.

Although 804G matrix is highly enriched in laminin-5, its ability to induce hemidesmosome assembly could be due to some other, presumably minor component of the matrix. For example, laminin-5 is purported to bind two other laminins (laminin-6 and laminin-7)(66). Thus to confirm the role of laminin-5 in 804G matrix induced hemidesmosome assembly we have taken two approaches. The first was to prepare a series of monoclonal antibodies against laminin-5 subunits. We then assayed the ability of these antibodies to inhibit hemidesmosome assembly in our model system. One antibody termed CM6 not only blocks hemidesmosome assembly in cells plated onto 804G matrix but also triggers internalization of hemidesmosomes in 804G cells themselves (37). This particular antibody recognizes the α3 subunit of laminin-5 and binds in the region of the G-domain of the intact molecule (37). This has led us to propose that the G-domain is involved in nucleation of hemidesmosome assembly and most likely contains the α6β4 integrin binding site (37).

In a second approach, we have assessed the ability of purified laminin-5 to induce hemidesmosome assembly. Our source of laminin-5 is the conditioned medium of 804G cells (67). Human corneal tissue explants, consisting of epithelial cells and connective tissue were incubated in medium containing the purified laminin-5 which is then incorporated not only into forming basement membranes but also into the preexisting basement membranes of the explants (67). This is intriguing since it implies that the basement membrane is quite dynamic and can be modified subsequent

to its deposition. Furthermore, there is an induction of hemidesmosome assembly in the epithelial cells of the explanted tissue. In more recent studies, we have plated keratinocytes onto surfaces coated with laminin-5 (68). Under these circumstances the keratinocytes are induced to assemble hemidesmosomes on the laminin-5 substrate (68).

Some patients suffering JEB have mutations in the genes encoding one of the subunits of laminin-5 (2,13-16). These mutations, in general, result in premature termination of message transcription, a recurring mechanism underlying the pathogenesis of those skin diseases whose targets are hemidesmosomal protein components. No mutated protein has been detected in the diseased cells and the absence of one laminin-5 subunit prevents assembly of the laminin-5 heterotrimer. One assumes that the absence of laminin-5 impacts hemidesmosome assembly. Laminin-5 is also the target of an autoimmune blistering disease, cicatricial pemphigoid (CP). Patients with CP have circulating autoantibodies against laminin-5 which are believed to be pathogenic in blister formation (10). Together these studies have provided important evidence that laminin-5 is involved in assembly of hemidesmosome and supports the results of our cell biological analyses of laminin-5 function.

Perspectives

Ten years ago we knew little about the composition of the hemidesmosome and few groups studied this cell-matrix junction. This situation has dramatically

changed. Major advances have been made in our understanding of hemidesmosome structure and function. Furthermore, we are beginning to elucidate the mechanisms underlying hemidesmosome assembly. One issue that intrigues us is how 804G and MCF-10A cells assemble their laminin-5 rich matrices (Fig. 4)(29,37,60). Both cell types lay down laminin-5 in remarkably intricate patterns. What determines these patterns? Are integrins involved? These and other unanswered questions concerning the ways hemidesmosome elements interact and the means by which the hemidesmosome is connected to cell signaling pathways are likely to be answered in the next few years.

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Figure legends

Figure 1. Hemidesmosome have a distinctive ultrastructural appearance. An electron micrograph of two hemidesmosomes along the basement membrane zone of the tongue. Note that each hemidesmosome has a dense cytoplasmic plaque, the site of keratin bundle (k) attachment, and sits on a sub-basal plate (small arrow) which occurs in the lamina lucida of the basement membrane. There is an anchoring fibril in the dermis beneath one of the hemidesmosomes (open arrow). Bar, 400nm.

Figure 2. This is a diagram of the hemidesmosome in which its major components are indicated. We have taken some liberties by indicating our own speculations on

protein-protein interactions in the hemidesmosome. Keratin bundles are represented by curved lines. IFAP300 is related to or is the same as a protein called HD1.

Figure 3. MCF-10A cells assemble hemidesmosomes in vitro. This electron micrograph shows a cross-section of an MCF-10A cell. Several hemidesmosomes occur at the site of cell-substrate interaction (arrows). These are similar in appearance to the hemidesmosomes in intact tissue (see Fig. 1). Bar, 300nm.

Figure 4. Human epidermal cells were plated onto the laminin-5 rich matrix of 804G cells in control medium (A-C) or medium containing the blocking α6 antibody GoH3 (D-F). 24 hr. later the cells were processed for double immunofluorescence using a hemidesmosomal antibody (A,D) and an antiserum that is specific for 804G laminin-5 (B,E). C,F, phase contrast. In A and B the epidermal cells have adhered to and spread on the laminin-5. In addition, hemidesmosomal elements in the cells co-localize with the laminin-5 in the matrix. In contrast in D and E there is no obvious co-distribution of hemidesmosomal elements in the cells and the underlying laminin-5, even though the cells have adhered to the matrix. In other words, the GoH3 antibody has blocked laminin-5/α6β4 integrin interaction, an event necessary for hemidesmosome assembly (37,60). Bar, 15μm

Figure 5. Keratinocytes were incubated in control and integrin antibodies for 15 min. at 37°C and then plated onto the laminin-5-rich matrix of 804G cells. In these studies

we used GoH3 antibody which inhibits α6 integrin function, antibody P1B5 which blocks α3 integrin and the non-inhibitory antibodies 3E1 against β4 integrin and P4C10 against β1 integrin. After 30 min. cell adhesion to the matrix was quantified colorimetrically using crystal violet. Adhesion is presented as a percentage of the adhesion observed using cells preincubated with 3E1 antibody. Adhesion of keratinocytes is partially inhibited (less than 20%) by the α3 integrin antibody P1B5 but not by the α6 integrin antibody GoH3. In combination these two antibodies inhibit cell attachment by more than 70%.